

**Role of Aluminum as a Toxic Element in Causing Parenteral Nutrition Associated
Cholestasis**

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By

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ABSTRACT

Parenteral nutrition (PN) is an essential life sustaining therapy for premature and critically ill infants. However, prolonged PN therapy can lead to life-threatening liver damage, and cause parenteral nutrition associated cholestasis (PNAC). There has been some recent evidence that aluminum accumulation in the livers of PN-fed subjects may lead to hepatic damage leading to liver injury. This dissertation aimed to investigate the role of aluminum as a toxic component of parenteral nutrition and as a risk factor in developing PNAC.

The project composed of two main studies. The objectives of the first study were:

1) Evaluate the early morphological changes in piglet liver after intravenous administration of aluminum chloride hexahydrate at a dose of 1500 $\mu\text{g/kg/d.}$; 2) Determine whether the morphological changes deteriorate further with increasing duration of exposure and whether these changes correlate with changes in biochemical markers of cholestasis; 3) Identify the appropriate imaging technique for studying the ultrastructural changes in the liver; 4) Determine if intravenous injection of high dose aluminum into neonatal piglets disrupts iron homeostasis in the liver.

The results showed that intravenous infusion of aluminum in neonatal piglets led to marked elevation in serum total bile acids, and transmission electron microscopy-energy dispersive microanalysis (TEM-EDX) was suitable in detecting the site of Al deposition in the liver and in demonstrating histopathological changes associated with Al infusion.

The objectives of the second part were to: 1) Investigate the role of aluminum as a toxic component of parenteral nutrition and as a risk factor in causing liver injury; 2) Evaluate the effect of reducing aluminum content of parenteral nutrition on liver iron homeostasis; 3) Investigate the effect of low aluminum PN and high aluminum PN (regular PN) on the mRNA

expression of Bsep and Mrp2.

The results showed that administration of PN solution with lower Al content led to reduced levels of serum and hepatic Al in low Al PN group compared to regular PN group. This reduction was associated with less histopathological changes in the liver. On the other hand, administration of regular PN in piglets led to decreased expression of transporter Mrp2.

This work suggests that reducing Al content in PN may reduce the development and severity of liver injury in the piglets.

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LIST OF ABBREVIATION

Al	Aluminum
ANOVA	Analysis of variance
ASCN/ASPEN	American Society of Clinical Nutrition / American Society of Parenteral and Enteral Nutrition
BHT	Butylated hydroxytoluene
BSEP	Bile salt export pump
dL	Deciliter
DMT-1	Divalent metal transporter 1
EDTA	Ethylenediamine tetracetic acid
EGTA	Ethylene glycol tetraacetic acid
FEC	Friend erythroleukaemia cells
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
FXR	Farnesoid X receptor
g	Gravity
HCP1	Heme carrier protein-1
ICP-MS	Inductively coupled plasma mass spectrometry
IL-1	Interlukin-1
IL-6	Interlukin-6
IRE	Iron responsive elements
IREG1	Iron-regulatory protein 1
IRP	Iron regulatory protein

IV	Intravenous
mmol/L	Millimole per liter
KCl	Potassium chloride
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MDR	Multidrug resistance
MgCl ₂	Magnesium chloride
min	Minutes
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRP2	Multidrug-resistance–associated protein
nm	Nanometer
Na Cl	Sodium chloride
NS	No significance
NTBI	Non transferrin-bound iron
NTCP	Sodium dependent taurocholate cotransporting polypeptide
OATP	Organic anion transporting polypeptide
OS	Oxidative stress
PN	Parenteral nutrition
P-value	Probability of significance
PNAC	Parenteral nutrition-associated cholestasis
PUFA	Polyunsaturated fatty acids
PXR	Pregnane X receptor

RIPA	Radioimmuno precipitation assay
ROS	Reactive oxygen species
rpm	Rotation per minute
QRT-PCR	Quantitative real time polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SRC	Saskatchewan Research Council
STXM	Scanning transmission x-ray microscope
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TEM-EDX	Transmission electron microscope-energy dispersive microanalysis
Tf	Transferrin
TfR	Transferrin receptor
TNF- α	Tumor necrosis factor- α
μ L	Microliter
μ mole	Micromole
WDX	Wave dispersive X-ray analyzer

1.0 Introduction

1.1 Rationale

Parenteral nutrition (PN) is the intravenous administration of complete and balanced nutrition given to patients who are seriously ill and are unable to tolerate enteral feeding. Since its introduction into clinical practice in the 1960s, parenteral nutrition has been associated with the development of parenteral nutrition associated cholestasis (PNAC). Prolonged parenteral nutrition (usually ≥ 3 weeks) is associated with complications affecting the hepatobiliary system, such as steatosis and cholestasis. While steatosis (fatty liver) is relatively more common in adults, cholestasis is more common in children (Payne-James and Silk, 1991; Quigley et al., 1993; Kelly, 1998). Parenteral nutrition associated cholestasis remains a significant problem in terms of not only incidence but also lack of effective treatment, the mortality, and morbidity associated with the illness. The occurrence of the illness is more frequent and severe in infants than in adults (Allard, 2002). The reported incidence of PNAC in children is quite variable from study to study. Data from epidemiological study reported that 7.4% of the 624 infants developed PNAC (Bell et al., 1986). Another study, the incidence of neonatal PNAC has been reported at 25% (Kubota et al., 2000), and associated mortality may range from 20% to 31% (Kubota et al., 2000; Ginn-Pease et al., 1985). Sondheimer et al., reported that cholestasis developed in 28 (67%) of the 42 infants receiving parenteral nutrition (Sondheimer et al., 1998). The incidence of PNAC might be as high as 84% in a study conducted on 31 infants receiving PN (Cohen and Olsen, 1981).

Although several biochemical parameters have been used to define this disorder for research purposes, a serum direct bilirubin ≥ 34 $\mu\text{mol/L}$ is considered the most commonly used marker after exclusion of other causes of cholestasis (Guglielmi et al., 2006). The common

histopathological features of PNAC are intracanalicular cholestasis and periportal fibrosis. Other histopathological findings include giant cell transformation, portal inflammation, acute cholangitis, hepatocellular damage and severe fibrosis (Benjamin, 1981; Hodes et al., 1982; Sokol, 1997; Kelly, 1998; Zambrano et al., 2004).

Despite the well documented association between PN administration and cholestasis, the precise etiology of PNAC remains unclear. Many researchers agree that the etiology of the disease is multifactorial. A number of risk factors that predispose children to PNAC are age-related risk factors including prematurity, lack of enteral feeding, and sepsis (Forchielli and Walker, 2003). Other important risk factors related to the PN components themselves are nutrient deficiencies, caloric load, and toxicity of nutrients (Forchielli and Walker, 2003).

Aluminum is a significant contaminant of PN component solutions such as calcium and phosphate salts (Sedman et al., 1985; De Vernejoul et al., 1985; Li et al., 2005). More than 81% of aluminum contamination in neonatal PN can be attributed to calcium gluconate (Mouser et al., 1998). When parenterally administered, aluminum bypasses the protective barrier of the gastrointestinal tract and may become deposited in bone, liver, spleen, kidney, brain, and other tissues (Yokel and McNamara, 2001; Priest et al., 1998). Approximately 40% of the intravenously infused aluminum is retained in adults and up to 75% is retained in neonates (Klein, 1995). Kidneys are the major excretory organ for aluminum, thus patients with reduced renal function and premature infants are at greatest risk for aluminum toxicity (Yokel and McNamara, 2001; Sedman et al., 1985; Advenier et al., 2003). Also, because of immature renal function, premature infants are more prone to aluminum toxicity due to their increased calcium and phosphorus requirements thus exposing them to more aluminum contaminants from the calcium and phosphorus salts (Advenier et al., 2003).

In addition to well established toxic effects of aluminum exposure on brain, liver, skeletal muscles, heart, and bone marrow (Galle, 1987; Bishop et al., 1997; Gilbert-Barness et al., 1998; Gonzalez et al., 2007; Al-Hashem, 2009; Mahdy, 2009), aluminum has also been implicated in altering cellular iron homeostasis. Disruption of cellular iron homeostasis can pose serious health problems. Elevated cellular iron concentration can induce oxidative stress to the cell and is linked to the pathogenesis of neurodegenerative diseases such as Alzheimer's, Parkinson's and heart-related diseases (Kell, 2009).

Despite the attempts to reduce the aluminum contamination in the PN solution, aluminum toxicity is still reported in many studies (Poole et al., 2008). Previous studies of aluminum exposure from PN solutions have reported aluminum intakes in the range of 10 - 60 µg/kg/day (Mouser et al., 1998; Popinska et al., 1999; Advenier et al., 2003), which significantly exceeds the recommendations set by the American Society for Clinical Nutrition, and American Society of Parenteral and Enteral Nutrition (ASCN/ASPEN) for safe aluminum infusion of 2 µg/kg/day (ASCN/ASPEN, 1991). These intake ranges also exceed the safe upper limit of 5 µg/kg/day Al recommended by Food and Drug Administration (FDA, 2000) and Health Canada (2011).

Research draws a correlation between aluminum loading and PNAC in neonates (< 28 days). Despite this relationship, the exact role of aluminum in the development of PNAC is unknown. No consistent histopathological changes are seen in the liver after infusion of Al. Moreover, such studies used less ideal and animal models (Klein et al., 1987; Klein et al., 1988).

Considering the significant aluminum contamination of the PN components, and the high risk of PNAC in neonates, the suggested relationship between aluminum contaminated PN solutions and the development of cholestasis needs further study. In a retrospective study in Saskatchewan, 31% of hospitalized infants with gastrointestinal failure who received parenteral

nutrition (PN) for 21 days developed cholestasis (Li et al., 2004). Although these PN solutions were contaminated with 21.6 µg/kg/d aluminum (compared to the recommended 'safe' level of 2 µg /kg/d for adults), its causal relationship to PNAC remains speculative.

In continuation of the previous work, a newborn piglet model was developed to study the effect of aluminum infusions on biochemical parameters and histopathological features of the liver. The newborn piglet is an excellent model for the human infant because of the comparative anatomy, physiology and metabolism, especially the similarities of the hepatobiliary system (Pond and Mersmann, 2000). In previous work conducted in our laboratory, piglets were orally fed and given daily intravenous injections of either a high dose (1500 µg/kg/d) or low dose (20 µg/kg/d) aluminum for 3 weeks and were compared with saline and PN controls (Li, 2004). After 3 weeks, the high aluminum group showed biochemical and histologic signs of cholestasis. In the low dose aluminum group, piglets had increased serum bilirubin levels but no histologic signs of cholestasis. In my work, I aimed to determine the early morphological ultrastructural changes in the liver that are associated with aluminum infusion and correlate those changes with biochemical markers of cholestasis namely the serum direct bilirubin and total bile acids. Furthermore, I aimed to identify an appropriate imaging technique(s) for the early detection of ultrastructural changes consistent with cholestatic changes of the liver. However, to reproduce the hepatic changes, it was first necessary to conduct a study using the high aluminum dose (1500 µg/kg/d) previously shown to cause cholestasis in piglets. This dose of aluminum was selected as being large enough to reproduce histologic and biochemical changes in the piglet's liver (Klein et al., 1987; Li et al., 2004) and, hence, allow for the identification of an appropriate imaging technique. The findings from this first study would then lead to the second major study of my PhD thesis research involving a clinical model of total parenteral nutrition infusion

containing high and low aluminum content.

The results obtained can better enable us to understand the morphological and biochemical changes during the infusion of aluminum as well as to determine the early changes associated with aluminum loading. These results might help to develop a better understanding on the role of aluminum in causing PNAC. This information would be very helpful in identifying the onset of the disease as well as reducing the incidence and severity of the disease.

1.2 Hypotheses and Objectives

The main hypothesis of my PhD thesis research was that high aluminum content PN causes early histopathological changes and elevated biochemical markers in the liver and lowering aluminum content would reduce the histopathological changes and lower the biochemical markers of tissue damage, and thus reduce the incidence and severity of PNAC. The primary objective of the present study is to evaluate the effect of low aluminum content PN compared to regular PN on the histopathological ultrastructure and biochemical markers in the piglet's liver. My secondary objective is to investigate the aluminum-iron interaction in the liver tissue and their role in causing liver injury.

This project is composed of two main studies; the first study is to examine the early morphological structural changes in the piglet's liver after daily intravenous infusion of high aluminum doses (1500 $\mu\text{g/kg/d}$) and correlate these changes with biochemical changes. Piglets will receive high aluminum dose for different durations of exposure and the degree of pathological changes in the liver and changes in biochemical markers of cholestasis will be correlated with the duration of aluminum exposure. The high dose of aluminum was previously shown to cause cholestasis in the piglet and use of this dose will allow a determination of the imaging technique that best identifies the ultrastructural changes in the liver with aluminum

exposure.

The second study will use a clinical model of total parenteral nutrition, the neonatal Yucatan piglet. The piglets will be administered PN with high and low dose aluminum for two weeks and the development of PNAC will be evaluated using the biochemical markers and the imaging technique identified in the first study. To test our hypothesis, this project utilizes a combination of morphologic and biochemical methods in order to elucidate the role of aluminum in causing liver injury in neonatal piglets.

2.0 LITERATURE REVIEW

2.1 Parenteral nutrition associated cholestasis

Since the introduction of parenteral nutrition (PN) in the 1960s, its use has become a vital tool in clinical practice. Parenteral nutrition is used frequently for nutritional support of clinically ill individuals with disorders of the gastrointestinal tract who cannot tolerate enteral intake (Burrin et al., 2003; Baserga and Sola, 2004). However, several studies have shown that prolonged use of PN is associated with liver and biliary system diseases including cholestasis, cholelithiasis, sepsis, and liver cirrhosis (Kelly, 1998; Briones and Iber, 1995). Parenteral nutrition associated cholestasis (PNAC) is defined as cholestasis that is associated with a prolonged duration of parenteral nutrition administration more than 2 weeks (Drongowski et al., 1989). Elevated serum direct bilirubin, total bile acids, and transaminases are the common biochemical presentation of PNAC. However, because this presentation is identical to other cholestatic liver diseases (Sokol, 1997; Kelly, 1998), therefore, PNAC can be accurately diagnosed only after other causes of extrahepatic cholestasis have been excluded, namely obstructive causes and metabolic liver diseases (Carter and Shulman, 2007; Guglielmi, 2008).

The frequency of parenteral nutrition associated cholestasis varies in studies from 7.4–84% (Beale et al., 1979; Cohen and Olsen, 1981; Bell et al., 1986; Sandhu et al., 1999). A number of studies indicate that approximately 40–60% of children on long-term PN will develop hepatic dysfunction (Kelly, 1998). In 1975, Rager et al. reported that nine of 15 premature infants who received PN have demonstrated cholestasis histologically. The nine infants had a serum total bilirubin greater than 8 mg/dL, the normal value for total bilirubin ranging 0.2-1.9 mg/dL (Catherine et al., 2003). The results of a retrospective study of neonates who received PN for at least one week reported that 15% of infants developed PNAC (Drongowski and Coran,

1989). In this study, the cholestasis was defined as a direct serum bilirubin greater than 2.0 mg/dL during the course of PN therapy. In another study, the overall incidence was 43% in infants receiving PN for 19 to 75 days (.Touloukian and Seashore, 1975). The incidence of PNAC closely related to duration of PN. For example, the overall incidence of cholestasis in 62 premature infants on PN was 23%, but infants receiving PN for more than two months has an incidence of 80% increasing to 90% in those on PN for more than 3 months. Serum direct bilirubin above 2.0 mg/dL was used as cut-off point to define cholestasis (Beale et al., 1979). In a more recent study, the author found that 25% of neonates with intestinal pathology who received PN for a mean of 18 days developed parenteral nutrition associated cholestasis (Arnold, 2002).

After more than 30 years, despite the well-documented association between PN administration and cholestasis, the precise etiology of parenteral nutrition associated cholestasis remains mystery (Carter and Shulman, 2007). Many researchers agree that the etiology of the disease is multifactorial. A number of risk factors that predispose children to PNAC are age-related risk factors including prematurity, lack of enteral feeding, low birth weight, sepsis, caloric overload, and toxicity of the PN components(Forchielli and Walker, 2003; Guglielmi et al., 2008) .

2.1.1 Potential risk factors

2.1.1.1 Prematurity and low birth weight

A relationship has been identified between the development of cholestasis in infants who are premature and of low birth weight. However, because many infants who need PN are likely to be premature and of low birth weight, it is difficult to decide whether prematurity and low birth weight are independent or associated risk factors. Premature are infants defined as those who are born before 37 weeks gestational age and are usually born with birth weight less than

2500 g. Beale et al., found that 50% of premature infants (n=62) receiving parenteral nutrition with birth weight less than 1000 g had cholestasis (direct bilirubin > 1.5 mg/dL), and 18 % at birth weight between 1000-1499 g and only seven % had cholestasis with a birth weight greater than 1500 g (Beale et al., 1979). The increased incidence of cholestasis in premature infants with low birth weight suggests that the disease may be related to the immaturity of neonatal biliary secretory system. The decreased size of the bile acid pool due to both reduced hepatic uptake and synthesis of bile acids make premature neonates more susceptible to the development of cholestasis (Afdhal and Smith, 1990; Gleghorn et al., 1989). Premature infants with a lower gestational age have shown to have elevated serum direct bilirubin concentrations (> 40 μ mol/L) and developed cholestasis (Pereira et al., 1981; Beath et al., 1996). Pereira et al. reported that the frequency of cholestasis was 1.4 % in the neonates who were born at a gestational age of > 36 weeks, and increased to 5.3% in infants who were born between 32 and 36 weeks, and reached 13.75 % for infants born before 32 weeks (Pereira et al., 1981). Although some studies have linked the development of cholestasis in infants to prematurity and low birth weight, more research is needed to identify whether prematurity and low birth weight are independent or associated risk factors.

2.1.1.2 Lack of enteral feeding

Lack of enteral feeding has a crucial role in the development of PNAC. The levels of cholecystokinin and other gastrointestinal hormones, such as glucagon, gastrin, and enteroglucagon are reduced in patients receiving parenteral nutrition compared to enterally fed (Greenberg et al., 1981). These hormones are involved in stimulating bile flow and contraction of gallbladder. A reduction in release of cholecystokinin, responsible for gallbladder contractility, resulted in formation of biliary sludge in the gallbladder, a common finding of

biliary stasis in infants with PNAC (Touloukian and Seashore, 1975). However, the results of some studies reported that using enteral feeding and increasing the levels of cholecystokinin hormone did not significantly reduce the incidence of PNAC (defined as direct bilirubin level > 1 mg/dL) (Teitelbaum, 2005; Demircan et al., 1998). Also, lack of enteral feeding contributes to intestinal hypomotility, which has been reported to promote bacterial overgrowth in the small intestine (Lucas et al., 1983). Bacterial overgrowth contributes to infections, bacterial translocation and endotoxin production, which in turn has the ability to downregulate bile acid transporting proteins (Trauner et al., 1998). In addition, bacterial overgrowth has the ability to convert chenodeoxycholic acid into a hepatotoxic bile acid lithocholic acid (Hofmann, 2004). However, in a retrospective study on 74 neonates received parenteral nutrition for more than 21 days, the author reported that lack of enteral feeding did not promote cholestasis (Beath et al., 1996), which implies that the importance of enteral feeding in the development of PNAC is still controversial and more research is need to be done.

2.1.1.3 Sepsis

Sepsis is one of the most common complications of total parenteral nutrition infusion in infants (Rannem et al., 1986). Beath et al. reported that surgical neonates had a 30 % increase in serum conjugated bilirubin concentrations during repeated episodes of sepsis (Beath et al., 1996). The mechanism of sepsis-induced cholestasis is unknown, but research has focused on the possible toxic effects of endotoxins on the hepatobiliary system. Endotoxins are released from the gram-negative bacteria like *Escherichia coli* during systemic infections, or may translocate from the gut into the portal circulation by binding to specific sites of the intestinal membrane after their release by enteral bacteria (Gonnella et al., 1992). In the liver, the amount of endotoxins may exceed the ability of Kupffer cells to detoxify them (Nolan, 1975), thus leading to their sequestration in hepatocytes (Utili et al., 1976; Gonnella et al., 1992) and causing direct hepatocellular injury. In response to bacterial inflammation, higher level of cytokines tumor necrosis factor (TNF) and interleukines-6 (IL-6), have been found in the rats receiving parenteral nutrition compared to the control group (Zheng, 2004). Moreover, treatment of animals with endotoxins or cytokines has been shown to cause down regulation of bile acid transporters (Green, 1996; Trauner et al., 1998; Denson et al., 2000), which are the crucial part of bile flow. However, in a study reviewing the medical records of 24 neonates with a clinical history of receiving PN who died at Yale –New Haven Children’s Hospital, the histological study of the liver samples found no significant relationship between parenteral nutrition induced-liver injury and sepsis (Zambrano et al., 2004).

In summary, it is likely that sepsis may contribute to PNAC through the involvement of endotoxins. However, the exact role of endotoxins as a causative factor in causing PNAC remains unclear.

2.1.1.4 Nutrients

2.1.1.4.1 Taurine deficiency

Taurine is an important amino acid for the conjugation of bile acids (Chesney et al., 1998). Premature infants have a decreased cystathionase level, an enzyme important for synthesis of taurine and cysteine from methionine (Zlotkin and Anderson, 1982). The inability to produce enough taurine favours the formation of hydrophobic and toxic glycine-conjugated bile acids. Taurine supplementation has been shown to maintain bile flow and bile acid secretion in guinea pigs (Guertin et al., 1993). On the contrary, Cooke et al found that taurine supplementation in premature infants did not show any effect on hepatic function (Cooke, 1984). Moreover, no correlation has been found between low plasma taurine concentrations and cholestasis, and no strong evidence suggests that returning plasma taurine concentrations to normal would prevent PNAC (Btaiche and Khalid, 2002). Recently, the formulation of some neonatal parenteral nutrition is now routinely supplemented with taurine (Forchielli et al., 1995; Adamkin et al., 1995; Thornton and Griffin, 1991). However, no conclusive evidence has shown that supplemental use of taurine reduced the incidence of cholestasis (Teitelbaum, 1997). More studies are needed to demonstrate its true usefulness.

2.1.1.4.2 Caloric load

Overfeeding of energy substrates either in the form of dextrose, amino acids, or fats, individually or combined has been reported to cause hepatic steatosis and cholestasis (Messing et al., 1992; Wagner et al., 1983). Excessive caloric intake is believed to promote fat deposition in the liver through stimulating insulin release, which in turn, promotes lipogenesis and inhibits the oxidation of fatty acids (Quigley et al., 1993). Reduction in total calories of parenteral nutrition solutions leads to improved jaundice and histologic features of cholestasis in adult patients (Messing et al., 1992). Excessive dextrose infusion may lead to steatosis but not to cholestasis in

adults (Btaiche and Khalidi, 2002). The administration of excessive calories has been associated with elevated transaminase levels and hepatic steatosis in humans and animals (Sheldon, 1978). In addition, it is noteworthy that providing a balanced dextrose and fat source of calories in PN formulations would reduce the incidence of steatosis. The recommended balanced PN should provide 70%-85% calories from carbohydrates and 15%-30% from fats (Miratallo et al., 2004).

However, now that dextrose-based PN formula is replaced with a more balanced dextrose-fat PN formulation there has been a reduction in the incidence of steatosis (Quigley et al., 1993). The role of caloric load in the development of PNAC is still unclear and not much research has been done to clarify this relationship.

2.1.1.4.3 Lipids

The parenteral lipid emulsion, Intralipid[®] (manufactured by Fresenius Kabi, Germany), a soybean-based lipid emulsion with egg yolk phospholipid, has been known for decades as the gold standard lipid emulsion in PN. However, this intravenous soybean-based lipid emulsion has been implicated in the development of hepatic complications (Chen et al., 1996; Zaman et al., 1997). Phytosterols, naturally occurring sterols in plants, equivalent of cholesterol, are found in soybean-based lipids. Unlike cholesterol, phytosterols are not converted to bile acids and they are much faster excreted by the liver (Boberg et al., 1990). Phytosterols has been postulated to have a deleterious effect on liver function (Clayton et al., 1993; Clayton et al., 1998). Lyer et al. (1998) reported that administration of daily injection of sterol into neonatal piglets had caused a significant increase in the serum bile acid levels. Furthermore, in isolated rat hepatocytes, phytosterols caused a significant inhibition in the secretory functions of the hepatocytes (Bindl et al., 2000). In children with biochemical evidence of PNAC, high serum levels of phytosterols have been reported (Clayton, 1993).

Another critical component of soybean based lipid emulsion have been implicated to play a role in the development PNAC are ω -6 PUFAs (ω -6 polyunsaturated fatty acids), linoleic and arachidonic fatty acid (Camandola et al., 1996; Park et al., 2001; Dichtl et al., 2002). The authors demonstrated that linoleic and arachidonic fatty acids increased the production of cytokines, inflammatory mediators, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6). Also, arachidonic acid is a substrate for proinflammatory mediators such as leuktriens B4 and C4, Thromboxane A2 and eicosanoids prostaglandin E2 (Tilley et al., 2001). These harmful effects led to the conclusion that increased levels of ω -6 PUFAs can cause inflammation (Calder et al., 2006).

Lately, Omeagven, fish oil based lipid emulsion receiving a lot of attention as a safe alternative intravenous lipid emulsion in PN. This fish oil based lipid emulsion, still not approved yet in Canada and USA to be used in clinical practice.

An animal study, using a fish oil-based lipid emulsion in PN instead of soybean-based lipid emulsion reported a reduced incidence of PNAC in neonatal piglets (Van-Aerde et al., 1999). The definition of PNAC was based on the bilirubin concentrations in serum. The average serum bilirubin levels were $17.2 \pm 2.4 \mu\text{mol/L}$ in the fish oil-based emulsion PN compared to soybean oil-based emulsion PN ($123 \pm 15.1 \mu\text{mol/L}$). In humans, case reports on the use of fish based lipid emulsions (i.e. Omegaven) reported significant improvements or complete resolution of cholestasis on the basis of histological features and serum direct bilirubin (Gura et al., 2006). Retrospective studies also supported the finding that Omegaven has a reversal cholestatic effect. Diamond et al. found that Omegaven restored liver function, reduced serum direct bilirubin concentrations and led to complete recovery from cholestasis in 9 of the 12 patients on PN with PNAC (Diamond et al., 2009). Also, in a case series study, the investigator observed that

Omegaven halted progressive PNAC in three of the four preterm infants with intestinal failure and PNAC (Cheung et al., 2009).

Although data evaluating the use of fish-oil based lipid emulsions in humans is limited, the fish oil-based emulsions are safer than soybean-oil based lipids and offer a promise in reducing the incidence of liver complications (Gura et al., 2006).

2.1.1.5 Toxicity of nutrients

Over the past decades, contamination of specific nutrients present or added to PN has been implicated in the pathogenesis of PNAC (Lavoie et al., 2005; Kelly, 2006). The implicated components include aluminum, copper and manganese.

2.1.1.5.1 Aluminum

Since 1980s, Al has been known as a contaminant in PN solutions (Klein, 1982). At that time, studies have estimated an increase in daily Al intake of 50-fold more than the present mean intake (Klein et al, 1984; Sedman et al., 1985; Klein, 1995). During that time, however, some changes in the components of PN have been made to reduce the total load of Al in the PN solutions. The casein hydrolysates, rich in Al, were replaced with crystalline amino acids that have lower Al content. Although these changes have reduced the overall Al content in the PN solutions, Al remains present in a significant amount in several components of PN solution, including calcium and phosphate salts (Sedman et al., 1985; De Vernejoul et al., 1985). Previous studies on Al exposure from PN solutions have reported that Al intakes in the neonates and children on PN range from 10 – 60 µg/kg/day (Mouser et al., 1998; Popinska et al., 1999; Advenier et al., 2003). More recently, Poole et al found that the calculated Al exposure in neonatal PN solutions in the range of 30 - 60 µg/kg/day (Poole et al., 2008). All of these studies exceeded the safe intake limit of 2.0 µg/kg/day of Al intake for adults recommended by the American Society of Clinical Nutrition/American Society of Enteral and Parenteral Nutrition

Joint Commission (ASCN/ASPEN 1991). Research to date on aluminum contamination and the development of hepatic dysfunction suggests a strong relationship. Much of the experimental research used non-neonatal models with aluminum doses far exceeding the level of contamination seen in PN. Hence, further research into the relationship between aluminum contaminated PN solutions and the development of PNAC in neonates and infants is therefore necessary.

2.1.1.5.2 Manganese

Manganese is an essential trace nutrient required as a catalytic cofactor for a variety of important enzymatic reactions in human physiolog, and it is one of the trace elements routinely added in parenteral nutrition admixture. In recent years, there is a growing concern that chronic overexposure to manganese in long-term parenteral nutrition therapy may lead to manganese toxicity or “hypermanganesemia”, which is often associated with hepatic and cerebral complications (Dickerson, 2001). A few studies have reported the effects of manganese toxicity in children receiving PN (Reynolds et al., 1994; Fell et al., 1996). In a study by Fell et al., 45 children (79%) of the 57 children receiving PN for more than two months had a higher plasma manganese level than the normal range. In the same study, 11 children (19.2%) of 57 had both cholestasis and hypermanganesemia. Cholestasis in this group was defined as total serum bilirubin > 12 $\mu\text{mol/L}$ (Fell et al., 1996). On the contrary, Beath et al. found no significant difference in the frequency of cholestasis between two groups who received either high or low manganese dosage in parenteral nutrition solutions (Beath et al., 1996). These results are supported by a recent study conducted on 54 infants receiving PN for four months. Of the 54 infants only 7 had high levels of serum conjugated bilirubin and manganese. The author concluded that no correlation was found between high blood manganese levels and cholestasis

(McMillan et al., 2008). No clear relationship between high levels of manganese and cholestasis has been established, and the existing data are contradictory. It is unclear if the elevated manganese causes cholestasis or is it merely elevated because it is not well excreted in the face of cholestasis?

2.1.2.5.3 Copper

Copper is an essential trace mineral and an integral part of several crucial enzymes in metabolism (Linder and Hazegh-Azam, 1996). Copper toxicity is very rare but chronic high intakes of copper are linked to Wilson's disease and liver cirrhosis (Araya et al., 2003). Copper is routinely included in the PN, and chronic high copper levels are hepatotoxic (Suita et al., 1999) and may predispose patients on PN to hepatic injury. A prospective study of children on PN found no correlation between cholestasis and copper levels (McMillan et al., 2008).

Cholestasis was defined as a serum conjugated bilirubin ≥ 2 mg/dL. Also, in a retrospective study of 28 cholestatic infants, their serum conjugated bilirubin > 2 mg/dL, who received PN and had 20 $\mu\text{g/kg/d}$ copper added to PN, only 2 (7%) of the infants had elevated serum copper levels with no correlation in their high serum copper levels to liver disease progression (Frem et al., 2010). Reducing copper in PN however, may cause copper deficiency. In 2004, Hurwitz et al. reported that an adult patient on PN with short bowel disease who developed cholestasis (defined as serum conjugated ≥ 2 mg/dL) and had the copper removed from his PN. The patient developed pancytopenia (reduction in red and white blood cells and platelets). The copper was added back to PN and the pancytopenia was resolved (Hurwitz et al., 2004). Copper is an important nutrient and it needs to be added in adequate amounts to prevent hepatotoxicity or deficiency.

2.2 Anatomy of the liver

The liver is the largest solid organ in the body, weighing about 1.5 kg in the adult (Lefkowitz, 2002). It lies in the right upper quadrant of the abdomen, inferior to the diaphragm and anterior to the stomach. The liver is unique amongst other organs. In addition to the supply of arterial blood from the hepatic arteries, it also receives blood from the portal vein, which drains venous blood from the digestive tract, pancreas and spleen. Thus, the liver is the main organ to metabolize all of the substances absorbed from the intestine. It is also the first organ to be exposed to toxic compounds absorbed from the gastrointestinal tract. The liver is an important metabolic organ; it acts as a transient nutrient store, transforms circulating metabolites and detoxifies harmful substances (Stieger and Meier, 1998; Suzuki and Sugiyama, 2000). Also, the liver functions as an exocrine organ by producing bile and by secreting bile into the duodenum to facilitate lipid digestion and absorption (Guyton, 2006).

The basic structural unit of the liver is the liver lobule (Figure 2.1), which is hexagonal in shape. The lobule is composed principally of many hepatic cellular plates. Each hepatic plate is usually two cells thick, and between the adjacent cells are the small bile canaliculi that empty into bile ducts (Guyton, 2006). Branches of the hepatic artery, portal vein, and bile ducts are commonly found at the corners of the lobules. In the center of the lobule, there is a central vein, which empties into the sublobular vein. Several sublobular veins converge to form hepatic veins that empty into the inferior vena cava. Plates of the hepatocytes radiate from the central vein to the perimeter of the lobule.

Blood flows from the hepatic artery and portal vein through the sinusoids and drains into the central vein. Sinusoids are lined by squamous endothelial cells and kupffer cells. The lining is discontinuous, allowing direct contact of hepatic cells with soluble components of blood, and permitting the easy exchange of macromolecules between blood and the hepatocytes. The

kupffer cells are derived from monocytes and are typical macrophages, capable of endocytosis of microbes and foreign particles (Guyton, 2006).

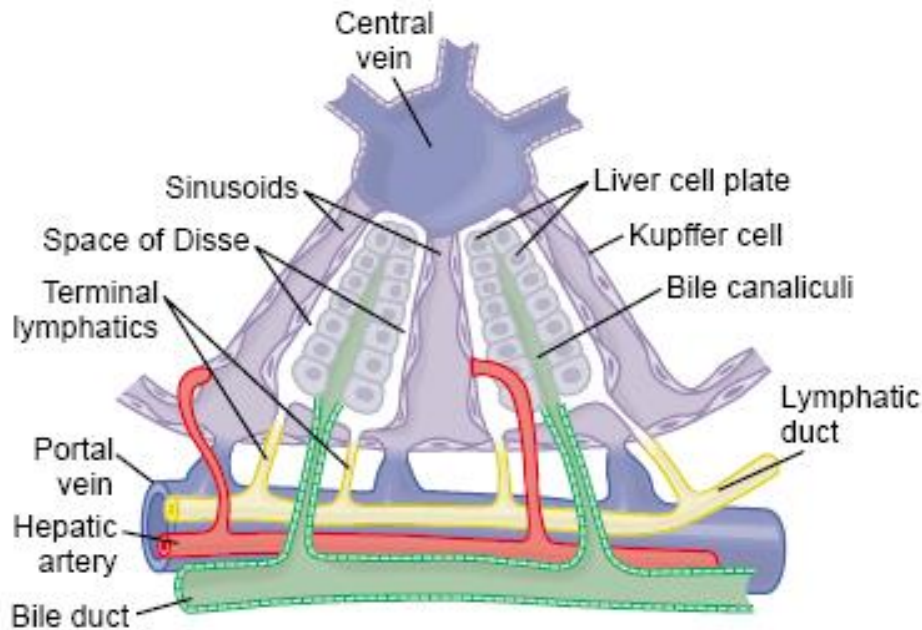


Figure 2.1. Composition of liver lobule.

(Source: Guyton, 2006) (Reprint permission obtained)

Hepatocytes constitute about 80% of the total cells in the liver. The multiple functions of the liver are primarily carried out by hepatocytes. The cytoplasm of hepatocytes often contains glycogen. Glycogen is mobilized when blood glucose concentrations are low. Hepatocytes synthesize and secrete various plasma proteins, such as albumin, prothrombin, fibrinogen and lipoproteins. Bile secretion is another important function of the hepatocytes. It synthesizes bile salts from cholesterol and secretes the salts into bile canaliculi. In addition to bile salts, cholesterol, phospholipids, bilirubin, water and electrolytes are co-excreted into the bile. Bilirubin is the metabolic waste of recycled red blood cells (Kuntz, 2006).

2.3 Bile formation, composition, and transport

2.3.1 Bile formation

Bile acids are synthesized from cholesterol in the liver. They are classified as primary and secondary bile acids. Primary bile acids are synthesized directly from cholesterol in the liver, and include cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas, secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA) are formed by metabolism of primary bile acids by colonic bacteria. The majority of the bile acids in humans are conjugated with taurine or glycine to form conjugated bile acids. Bile acids are present as sodium salts and usually referred to as bile salts (Chiang, 1998). These bile salts are then secreted across the canalicular membrane into bile canaliculi, which are small channels formed by half tubules from the apical surface of two adjacent hepatocytes (Guyton, 2006). The bile canaliculi drain into bile ductules, which empty into the terminal branches called the terminal bile ducts (Saxena et al., 1999). Then, the bile flows into the right and left hepatic ducts that ultimately drain into the common hepatic duct (Boyer and Nathanson, 1999). Bile is stored in the gallbladder and subsequently secreted into the common bile duct via the cystic duct. In its course through the bile ducts, a second portion of liver secretion is added to the initial bile. This additional secretion is a watery solution of sodium and bicarbonate ions secreted by secretory epithelial cells (cholangiocytes). Cholangiocytes deliver fluids and electrolytes in response to hormonal secretion (primarily secretin) released by the duodenum into the portal bloodstream after stimulation by acidic pH, fatty acids, and bile acids (Kim et al., 1979).

2.3.2 Composition of bile

The normal adult liver secretes approximately 600 to 800 mL of bile a day. Dissolved solids are 3% of bile by weight, and bile acids are the predominant organic solute at a biliary concentration of 20 to 30 mmol/L. In addition to bile acids, phospholipids and cholesterol are

the next two most prevalent components, and they make up respectively 17% and 3% of the solutes in bile (Table 2.1). Other components in the bile are present in small amount such as steroids, vitamins, drugs, and xenobiotics. Bilirubin, present at a concentration of 0.2 mmol/L constitutes 1% of the bile volume. Glutathione is present at relatively high concentrations of 5 to 10 mmol/L in the cytosol of hepatocytes and used in many reducing reactions and glutathione and glutathione conjugates actively secreted into bile. Inorganic salts are present in concentrations similar to those in plasma.

Table 0.1. Composition of bile.

Component	%
Bile salts	41
Electrolytes	31
Phospholipids	17
Proteins	7
Cholesterol	3
Bilirubin	1

(Modified from Vlahcevic, 1996)

2.3.3 Bile acid transport

2.3.3.1 Enterohepatic circulation

Bile acids, once produced in the liver, transported across the canalicular membrane of the hepatocytes into the bile and stored in the gallbladder. After each meal, bile acids are released into the intestinal lumen, where they are efficiently reabsorbed by the enterocytes and transported back to the liver via portal blood for re-excretion into the bile. This process is known as enterohepatic circulation of the bile (Chiang, 1998), and it is the main determinant of bile

flow. The hepatocyte depends on the recirculation of bile acids to maintain the bile acid pool, which is 3 to 4 g in adult humans. More than 95% of the bile acids excreted by the hepatocytes ultimately returned to the liver and resecreted into bile, and only 5% is lost into the feces. The daily loss of bile acids compensated by *de novo* synthesis in the liver and thus, a constant bile acid pool is maintained (Meier and Stieger, 2002). Bile acid transporters play important roles in this transport process. The canalicular excretion of bile acids into bile against concentration gradient is the major driving force of normal bile flow (Trauner and Boyer, 2003).

2.3.3.2 Hepatic bile acid transport

2.3.3.2.1 Hepatic bile acid uptake

The hepatocytes are polarized epithelial cells with basolateral (sinusoidal) and apical (canalicular) membrane domains. Hepatocytes take up bile acids through the basolateral membrane, which is in direct contact with the portal blood plasma, and excrete bile acid at the canalicular membrane into the bile (Boyer, 1980). Unconjugated bile acids are uncharged molecules under physiological pH and can pass through the cell membrane via passive diffusion. However, majority of the bile acids are conjugated with taurine or glycine and cannot cross the cell membrane and need active transport mechanisms for cellular uptake (Meier, 1995). Two bile acid transporters, sodium-dependent taurocholate transporter (NTCP) and organic anion transporting polypeptide (OATP) are responsible for basolateral bile acid transport into the hepatocytes (Table 2). The sodium-dependent taurocholate transporter co-transport two Na^+ ions down its concentration gradient into the hepatocytes along with one molecule of conjugated bile acid. The trans-membrane Na^+ gradient is in turn maintained by the $\text{Na}^+ - \text{K}^+$ -ATPase (Kullak-Ublick et al., 2000). Na^+ -dependent bile salt uptake pathway accounts for 80% of the total taurocholate uptake and is considered as the major bile acid transport system located at the

basolateral membrane (Meier and Stieger, 2002). The Na^+ -independent bile salt uptake is mediated by several members of the OATP family. Besides conjugated and unconjugated bile acids, many amphipathic organic compounds such as bilirubin, selected organic cations and numerous drugs are also taken up by these transporters (Meier et al., 1997).

Table 2.2. Function of hepatocyte and cholangiocyte transporters in bile secretion.

Name	Abbreviation	Location	Function
Sodium–potassium ATPase	Na ⁺ /K ⁺ – ATPase	Basolateral membrane	Removal of 2 Na ⁺ in exchange for 3 K ⁺ , thus creating a favorable Na ⁺ Gradient
Na ⁺ -taurocholate cotransporter	NTCP	Basolateral membrane	Hepatic uptake of bile acids
Organic-anion–transporting polypeptide	OATP	Basolateral membrane	Multispecific carriers for sodium-independent uptake of bile salts, organic anions, and other amphipathic organic solutes from portal blood
Multidrug-resistance–associated protein	MRP2	Canalicular membrane	Mediates ATP-dependent multispecific organic-anion transport (e.g., bilirubin diglucuronide) into bile; contributes to bile-salt–independent bile flow
Multidrug-resistance-3 P-glycoprotein (phospholipid transporter)	MDR3	Canalicular membrane	ATP-dependent translocation of phosphatidylcholine from inner to outer leaflet of membrane bilayer
Canalicular bile-salt–export pump	BSEP	Canalicular membrane	ATP-dependent canalicular transport of bile acid
Glutathione transporter	GSH transporter	Canalicular membrane	Glutathione transport into bile; stimulates bile flow independent of bile salts
Chloride channel	Cl ⁻ channel	Canalicular membrane	Facilitates chloride entry into bile
Potassium channel	K ⁺ channel	Basolateral membrane	Determines membrane potential
Cystic fibrosis transmembrane regulator	CFTR	Apical (luminal) membrane	Chloride channel; facilitates chloride entry into bile
Chloride–bicarbonate anion exchanger isoform 2	AE2	Apical (luminal) membrane	Facilitates bicarbonate secretion into bile and contributes to bile flow independent of bile salts

(Modified from Trauner et al., 1998)

2.3.3.2.2 Canalicular bile acid excretion

Several members of the ATP-binding cassette (ABC) transporter family, driven by ATP hydrolysis, are responsible for transporting bile acids and other organic compounds across the canalicular membrane against their concentration gradients. The bile salt export pump (BSEP), the major canalicular bile acid transport system, is responsible for monovalent bile acid (eg.taurocholate) transport at the canalicular membrane (Childs, 1995). Divalent bile acids with two negative charges such as sulfated taurocholates are transported across the canalicular membrane by another ABC transporter called multidrug-resistant associated protein MRP2 (Buchler, 1996). MRP2, is also known as the canalicular multispecific organic anion transporter (MOAT) (Cui, 1999; Konig et al., 1999), mediates the excretion of glutathione conjugates and bilirubin. Defects in MRP2 gene result in Dubin-Johnson Syndrome characterized by hyperbilirubinemia and hepatic accumulation of various endogenous and exogenous organic metabolites (Keitel et al., 2000).

After bile acids are pumped into the bile, they stimulate phospholipid and cholesterol secretions into the bile, followed by a passive inflow of water (Oude Elfrink et al., 2000). The excretion of phospholipids is mediated via the MDR3, and the major phospholipid in the bile is phosphatidylcholine (Smit, 1993; Langheim et al., 2005). Bile acids, phospholipids and cholesterol are three major organic solutes of the bile and once secreted, they form mixed micelles to reduce their toxicity to the bile duct. Normal bile formation depends largely on balanced secretion of these constituents. Impaired secretions will disrupt the bile flow and result in cholestasis.

2.4 Bilirubin metabolism

Bilirubin is a catabolic by-product of hemoglobin from hemolyzed red blood cells. The production of bilirubin from hemoglobin takes place in the reticuloendothelial cells. First, heme

is converted to biliverdin by hemeoxygenase, and then biliverdin reductase catalyzes the conversion of biliverdin to bilirubin (Figure 2.2). Unconjugated bilirubin is transported in the plasma tightly bound to albumin. In the liver, despite tight albumin binding, the unconjugated bilirubin dissociates from albumin in the sinusoid and diffuses across the unstirred water layer at the surface of the hepatocyte (Ananthanarayanan et al., 2001). The mechanism for transport of bilirubin across the plasma membrane into the hepatocyte involves transport proteins, such as the organic anion transporter (Knisely, 2004). In the smooth endoplasmic reticulum of the hepatocyte, unconjugated bilirubin is catalyzed by bilirubin uridine diphosphate glucuronyl transferase to form bilirubin diglucuronide and monoglucuronide. Biliary canalicular excretion of conjugated bilirubin is mediated by multi-drug resistance protein-2 (MRP2) located in the canalicular membrane with the apical region of the hepatocyte (Kuijck et al., 1997). Biliary excretion of glucuronide is the rate-limiting factor in the transport of bilirubin from plasma to bile. In the small bowel, bilirubin diglucuronide is not absorbed. Further metabolism occurs mainly in the large bowel, bacterial β -glucuronidases hydrolyse the conjugated bilirubin, which is then reduced to urobilinogens and urobilin which are excreted in the stool. Most of urobilinogen is excreted in feces and the rest is absorbed and re-excreted by the liver (enterohepatic circulation) and kidneys. In case of hepatocellular dysfunction, re-excretion by the liver is impaired and more is excreted in the urine.

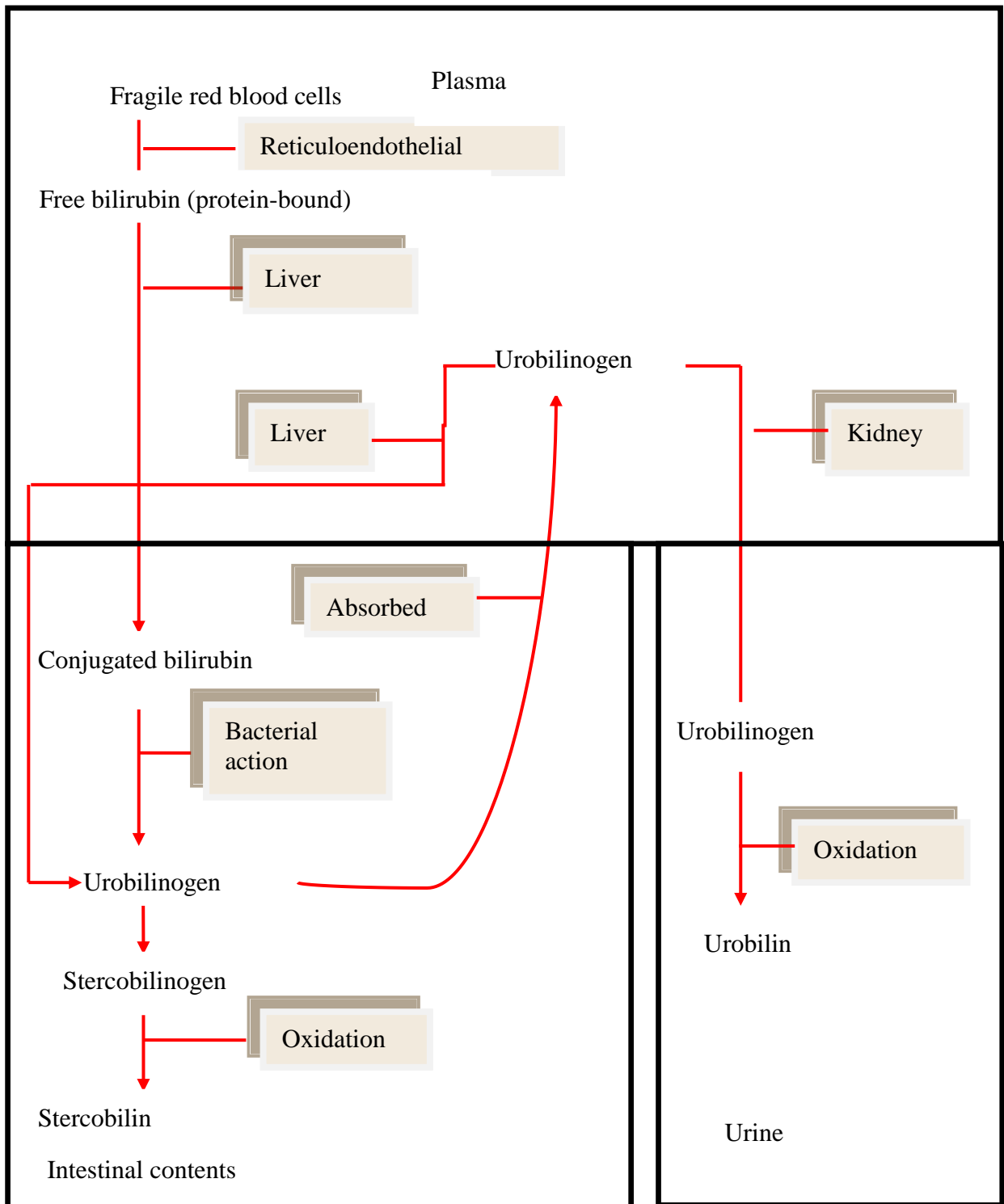


Figure 2.2. Bilirubin formation and excretion

Modified from Guyton 2008

2.5 Cholestasis

Cholestasis is defined as the cessation or reduction of bile flow in a pathological situation. It results from either a functional defect in bile formation at the level of the hepatocyte or from impairment in bile secretion and flow at the bile duct level (Trauner et al., 1998; Koopen et al., 1998). Cholestasis can be divided into extrahepatic cholestasis and intrahepatic cholestasis, which includes obstruction of the intrahepatic bile ducts and hepatocellular cholestasis (Erlinger et al., 1999). Despite the different causes of cholestasis, each of these diseases results in marked functional impairment of hepatocellular uptake and canalicular excretion of bile salts and various other organic anions (Moseley et al., 1996; Simon et al., 1996; Bolder et al., 1997). Other changes in liver function and structure also occur in many cholestatic diseases. These include changes in cytoskeletal architecture (Song et al., 1996) alterations in tight junctional permeability (Rahner et al., 1996) and a decrease in the fluidity of the canalicular membrane.

Cholestasis is defined as serum conjugated bilirubin concentration $\geq 34 \mu\text{mol/L}$ (Teitelbaum et al., 1997). The extent and duration of elevated serum direct bilirubin may predict severity and mortality in patients with cholestasis (Beath et al., 1996; Teitelbaum et al., 1996).

Other biochemical parameters such serum alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) are also sensitive markers for hepatobiliary disease, but they lack specificity because their levels may be elevated in other diseases (Kumpf, 2006). The values for ALP and GGT were normal in cholestatic infants. Serum concentration of aspartate aminotransferase (AST) and alanine transferase (ALT) may also be elevated after onset of cholestasis or jaundice (Vileisis et al., 1981; Ginn-Pease et al., 1985).

Despite the variation in the features of the histopathological findings of cholestasis among patients, there are common pathologic features like presence of bile thrombi in bile

canaliculi and periportal fibrosis. Other features also have been seen in patients with PNAC include giant cell transformation, portal inflammation, acute cholangitis, hepatocellular damage and severe fibrosis (Benjamin, 1981; Hodes et al., 1982; Sokol, 1997; Kelly, 1998; Zambrano et al., 2004).

2.5.1 Pathophysiology and molecular mechanisms of cholestasis

2.5.1.1 Extrahepatic cholestasis

Extrahepatic cholestasis results from the physical obstruction of the bile ducts. In an animal model of ligated common bile duct, the inability of bile to enter the intestine results in an increase in bile acid concentrations in the bile duct lumen and within the hepatocytes (Setchel et al., 1997). In addition, it causes dilatation of bile canaliculi with a loss of microvilli and alteration of intracellular junctions. Consequently, these alterations lead to regurgitation of bile components into blood (Accatino et al., 1981). As a result, expression of Ntcp and Oatp1 at the basolateral membrane is reduced (Gartung et al., 1996) limiting hepatocellular uptake of toxic bile acids. Short-term common bile duct ligation also results in relocation of Mrp2 from the apical membrane to a subapical vesicular compartment (Paulusma et al., 2000). There are also specific mechanisms that decrease canalicular efflux. The expression of Mrp2 (Lee et al., 2000) is significantly decreased, and this impairs secretion of sulfated bile acids as well as conjugated bilirubin (Moseley et al., 1996). However, Bsep is reduced by only 20%, and therefore some efflux of bile acids into the biliary space continues (Lee et al., 2000). This serves to protect hepatocytes from accumulating toxic levels of bile acids. In addition, there is up-regulation of Mrp1 and Mrp3 at the basolateral membrane of the hepatocytes (Ogawa et al., 2000; Paulusma et al., 2000; Hirohashi et al., 2000; Hirohashi et al., 1998) leading to the efflux of bile acids into plasma, thus preventing further hepatocellular toxicity. In the bile duct, there is proliferation of cholangiocytes (Alpini et al., 1988) as well as an increase in the expression of the apical sodium-

dependent bile salt transporter (ASBT) at the apical membrane of the cholangiocytes (Lee et al., 1999). This provides a mechanism for the removal of conjugated bile acids from the lumen of the bile duct. Furthermore, up-regulation of Mrp3 expression at the basolateral membrane of the cholangiocytes results in efflux of bile acids (Soroka et al., 2001). Finally, there is a marked increase in ductal HCO_3^- secretion resulting from up-regulation of secretin receptors. Therefore, although ductal obstruction prevents net bile flow, the compensatory mechanisms result in limitation of bile acid accumulation within hepatocytes and the duct lumen and profound dilation of the obstructed duct. It is noteworthy that prolonged biliary obstruction leads to bile ductular proliferation, fibrosis, and secondary biliary cirrhosis in animals and humans (Erlinger et al., 1999).

2.5.1.2 Intrahepatic cholestasis

Obstruction of intrahepatic bile ducts and alteration of bile secretion by hepatocytes (hepatocellular cholestasis) are the main causes of intrahepatic cholestasis.

In the obstructive intrahepatic bile duct, the mechanism of cholestasis is the same as in extrahepatic obstruction with exception of cystic fibrosis disorder, in which the function of cystic fibrosis transmembrane conductance regulator (CFTR) is impaired and associated with decreased secretion of chloride and bicarbonate (Roy et al., 1982). The decreased ductal bile flow leads to mucous precipitation and obstruction of small bile ducts followed by ductular proliferation, inflammation, and ultimately leads to focal biliary fibrosis (Roy et al., 1982). Hepatocellular cholestasis is subdivided into genetic and acquired cholestasis. There are three genetic forms of cholestasis have been identified and named progressive familial intrahepatic cholestasis type 1, 2 and 3 (PFIC-I, II and III). Progressive familial intrahepatic cholestasis (PFIC-I) is an autosomal recessive disorder caused by mutation in transporter gene (putative aminophospholipid transporter) (FIC1/ATP8B1) (Zollner and Trauner, 2008). Progressive familial intrahepatic

cholestasis I patients develop liver cirrhosis in early childhood (Bull, 1998). Children with PFIC-I usually present with elevated levels of serum bile acids, bilirubin, and transaminases in the neonatal period and they rapidly progress to end-stage liver disease requiring liver transplantation at an early age (Zollner and Trauner, 2008). In PFIC-II patients, a subtype of Byler disease is clinically indistinguishable from PFIC-I, mutations of the bile salt export pump gene (BSEP/ABCB11), which encodes BSEP, the major canalicular bile acid export system, causes PFIC-II (Strautnieks et al., 1998). Immunostaining of the liver of these patients shows an absence of BSEP at the canalicular membrane (Jansen et al., 1999). These patients have a near-total absence of bile acids from the bile and consequently develop bile acid hepatotoxicity because of accumulation within the hepatocytes. The third type of progressive familial intrahepatic cholestasis, PFIC-III, is caused by a mutation of the MDR3 gene, and leads to the absence of phospholipid secretion in the bile, which is important for forming micelles with bile acids. Its absence increases bile acid concentration in the biliary tract, which consequently leads to injury and proliferation of bile ductules (Deleuze et al., 1996).

Besides the genetic defects, cholestasis also develops during certain pathological conditions. Liver inflammation and sepsis caused by drugs or infection are often associated with cholestasis (Rodriguez-Garay, 2003). In this type of cholestasis, also called inflammatory cholestasis, an acquired cholestasis, the mechanism mediated primarily by cytokines, such as tumor necrosis factor- α (TNF- α), Interlukin-1 IL-1, and Interlukin-6 (IL-6), that is released in response to bacterial lipopolysaccharide, stimulation of activated macrophages and kupffer cells.

The effect of these cytokines has been extensively studied in several experimental models. Injection of lipopolysaccharide (LPS), a gram-negative bacteria cell wall component, induces inflammation and cholestasis in rodents. These animals showed reduced Mrp2

expression and hyperbilirubinaemia, indicating downregulation of hepatic transporters during inflammation might be the cause of cholestasis in these animals (Rodriguez-Garay, 2003). Extensive studies have made it clear that nuclear receptors are key regulators for the expression of these transporter genes. For example, PXR regulates the efflux hepatic transporters such as MDR1 and MRP2 (Geick et al., 2001; Kast et al., 2002) which are crucial for normal bile formation whereas, FXR regulates the canalicular bile acid transporters BSEP, Mrp2, and the uptake hepatic transporter NTCP (Ananthanarayanan, 2001; Kast et al., 2002). Studies from several groups have shown that some nuclear receptor expression is decreased dramatically by cytokines produced during inflammation, which is suggested to be the major cause of reduced expression of certain hepatic transporters (Teng and Piquetter-Miller., 2005; Cheng et al., 2005; Xu et al., 2004; Kim et al., 2003). Further studies in patients with hepatic inflammation and sepsis may be required to demonstrate the contribution of nuclear receptor down regulation to cholestasis. The cholestatic effects of endotoxin and endotoxin-induced cytokines not only have a role in the pathogenesis of inflammatory-induced cholestasis, but it may explain defects in hepatobiliary excretory function during total parenteral nutrition (Moseley et al., 1997).

Another type of acquired cholestasis is intrahepatic cholestasis of pregnancy (ICP). The cause of ICP is hypothesized to result from the hormonal changes during the pregnancy. Several hormones including estrogen and progesterone have been reported to show inhibitory effect on canalicular transporters. This form of intrahepatic cholestasis is reversible and is usually resolved after delivery (Riely and Bacq, 2004).

2.5.1.2.1 PN and hepatic transporter proteins

During the past decade, the pathogenesis of several forms of cholestasis has been attributed to mutations in hepatobiliary transporter proteins especially the canalicular transporters

(efflux transporters) and their behaviour during the administration of parenteral nutrition. The efflux transporters are members of the ATP- binding cassette (ABC) transporter proteins (Trauner, 1998). These transporters including MDR1, MDR3 (Mdr2 in rodents), MRP2, and BSEP are responsible for the transport of bile components from hepatocytes into bile canaliculi (Oude Elferink et al., 1995; Muller et al., 1996). The MDR1 transports chemotherapy drugs, MDR3 transports phosphatidylcholine, MRP2 transports conjugated bilirubin and BSEP transports bile salts from the hepatocytes to the bile canaliculi.

Studies on the effect of parenteral nutrition on the hepatobiliary transporter proteins have shown that the expression of both human MDR3 (orthologue of rodent Mdr2) and mdr2 in mice were reduced when treated with total parenteral nutrition whereas bsep expression in mice is increased with administration of PN (deVree et al., 1999; Tazuke et al., 2004). In another study, the expression levels of biliary efflux transporters Mdr2, Mrp2, and Bsep were decreased following the administration of PN without fat compared to PN with fat (Nishimura et al., 2005).

However, few animal models of PN including mice and rats have been used to study molecular mechanisms of PN-induced liver injury (deVree et al., 1999; Tazuke et al., 2004; Tazuke and Teitelbaum, 2009; Nishimura et al., 2005).

2.6 Aluminum metabolism

Al is ubiquitous, the third most abundant element after oxygen and silicon, in the earth's crust. Humans are exposed to aluminum compounds by eating food, drinking water, ingesting drugs containing Al like antacid and aspirin, breathing air or through skin contact by using antiperspirants. In foods, the concentration varies, depending upon the product, the type of processing, and the geographical origin (Pennington et al., 1987). The average intake of Al in a normal diet believed to be between 1 and 20 mg/day (Lione, 1983). The highest sources of Al in

foods are herbs and tealeaves; 8 oz of tea would add 1-3 mg Al to the diet (Greger and Sutherland, 1997). Infant milk formulas also contain Al. In cow's milk-based formula, the Al ranged from 0.03 mg/L to 0.2 mg/L whereas in soya-based formulas, the Al ranged from 0.64 mg/L to 1.34 mg/L (Baxter et al., 1990). In drinking water, Al concentration does not exceed 0.1 mg/L. The absorption of Al via the gastrointestinal tract is believed to be less than 1% (Greger and Sutherland, 1997). In Wistar rats, the gastrointestinal absorption of Al was 0.1 percent of the administered dose (Jouhanneau et al., 1997).

Although much has been published on intestinal absorption of Al, the mechanism by which Al passes through the intestinal wall is not completely understood (Yokel 1994; Exley et al., 1996; Lione, 1985; Priest, 1993; Wilhelm et al., 1990). However, the suggested mechanism of Al absorption in the gut includes both paracellular passage of Al through tight junctions by passive processes (diffusion) and transcellular passage across intestinal cells, involving passive, facilitated, and active transport processes (Van der Voet, 1992). In the blood, 90% of Al is bound to transferrin (Harris and Messori, 2002). Other elements are believed to be bound to Al include citrate and, to a lesser extent, phosphate. Al uptake by organs is believed to occur from the Al bound to transferrin (Ganrot, 1986). However, transferrin is not the only means by which Al enters the cells. DeVoto and Yokel (1994) assumed that the uptake of Al by cells is via diffusion or via pinocytotic uptake of extracellular fluid.

2.6.1 Aluminum accumulation in the body

Al occurs normally in all body tissues of humans (Ganrot, 1986). The total concentration of Al in the healthy human body is approximately 30–50 mg (Alfrey, 1984; Cournot-Witmer et al., 1981; Ganrot, 1986). Approximately 50% of total body Al is found in the skeleton and 25% in the lungs (Ganrot 1986).

In the literature, there are no unified standard normal values of Al in the human serum. In a study conducted on 71 office employees, the serum Al levels ranges from 1-3 µg/L with a mean of 2.67 µg/L (House, 1992). A literature review of studies published in the previous 30 years suggested that the serum concentration of Al ranged from 0.5 to 8 µg/L and 2 to 8 µg/L in whole blood (Caroli et al., 1994). In another study, the serum Al values in the control group ranged from 0.03 to 3.12 µg/L with a mean of 0.99 ± 0.97 µg/L, whereas in dialyzed patients were from 0.5 to 45.1 µg/L (mean of 4.75 ± 9.23 µg/L) (Razniewska et al., 2005). In the normal newborn, the average concentration of serum Al was reported at 5.17 µg/L (Sedman et al., 1985), and 8 to 12 µg/L (Litov et al., 1989). In normal full-term infants, the mean serum Al concentration was 7.8 µg/L, whereas, in preterm infants at gestational age 28 to 32 weeks, the average serum Al concentration was 13.2 µg/L (Bougle et al., 1992). In premature infants who received parenteral nutrition, the serum Al concentration averaged 37 µg/L (Sedman et al., 1985). In another study, the mean serum Al concentration was 15.9 µg/L in those who received parenteral nutrition compared to 8.9 µg/L, which were enterally fed (Bougle et al., 1992).

Aluminum is unequally distributed throughout the body tissues. In normal adults, tissue Al concentrations in lung, bone, liver, kidney, and brain were 20, 1 to 3, 1, 0.5, and 0.35 mg/kg respectively (Nieboer et al., 1995). In a review of the literature for the past 30 years, similar values were obtained; 2.21 to 15.3 mg/kg in the lung, 1.0 to 2.45 mg/kg in the liver and 0.55 to 1.31 mg/kg in the kidneys (Caroli et al., 1994). With increased age and accumulation of Al, concentrations of Al were 25-50 mg in bone, 20 mg in lung, and 9 to 24 mg in other soft tissues (Keith et al., 2002). In adult patients receiving parenteral nutrition, Al concentration in bone tissues of six patients was 14 to 265 mg/kg, and in the plasma was 98 to 214 µg/L (Klein et al., 1982). In animals, the results of a study conducted on piglets receiving parenteral nutrition for

21 days reported the mean Al concentration in the liver tissues at $0.90 \pm 0.37 \mu\text{g/g}$ and in controls was $0.05 \pm 0.06 \mu\text{g/g}$ (Li, 2004).

2.6.2 Aluminum excretion

The primary route of Al elimination is via the kidneys, and secondarily via bile. The kidneys excrete > 95 % of eliminated Al, presumably as a citrate. Humans who consume a normal diet with no medications and who have normal renal function excrete less than 50 μg Al/day in urine (Greger and Sutherland, 1997). Patients taking Al antacids in the diet had only a 3-fold increase in urinary Al levels (Gorsky et al., 1979), suggesting that most of the Al hydroxide was not absorbed and was excreted directly into the feces. Fecal Al represents unabsorbed Al as well as Al excreted via bile.

2.6.3 Aluminum concentrations in parenteral nutrition solutions

One such proposed etiological factor implicated in the development of PNAC is the presence of various contaminants in PN solutions. Aluminum is a non-essential element, and has been known as a significant contaminant in components of PN solutions, including calcium and phosphate salts (Stedman et al., 1985; De Vernejoul et al., 1985). Aluminum concentrations in neonatal PN solutions have been found to exceed the safe level (2 $\mu\text{g/kg/day}$) recommended for adults by American Society for Clinical Nutrition and American Society of Parenteral Enteral Nutrition (ASCN/ASPEN, 1991). In a study conducted on a group of 26 preterm infants receiving parenteral nutrition, the level of Al in PN ranged from 4.1 to 7.2 $\mu\text{mol/L}$, representing an average intake of 16.7 $\mu\text{g/kg/day}$ which significantly exceeds the ASCN/ASPEN recommendation for safe Al infusion (Moreno et al., 1994). Also, a retrospective study of 1003 infants on PN, the total daily Al ranged 30 – 60 $\mu\text{g/kg/d}$ in those weighing < 3 kg. The calculated Al exposure in these infants was approximately 6-12 times greater the recommended

safe levels (Poole et al., 2008). Although many attempts have been made to reduce the overall Al content in the components of PN, the currently available parenteral products used to make PN solutions for neonatal patients still contain amounts of Al exceeding the recommended safe level of Al.

2.6.4 Aluminum impact on hepatobiliary system

Aluminum toxicity is well documented but the mechanism of action is still poorly understood (Han and Dunn, 2000). Aluminum present in PN has been shown to accumulate in serum, body tissues, and urine of children and infants receiving parenteral nutrition solutions. The hepatic Al levels in five children who received PN for 18 to 33 months were five to 27 times higher than normal levels. In addition, they had elevated levels of serum total and direct bilirubin. Moreover, the histopathological observation of the liver showed that all of the five children had morphological changes such as bile duct proliferation, inflammatory cells and periportal fibrosis (Klein et al., 1984). In animals, Al present in parenteral nutrition solution accumulated in the liver of rats and produced hepatobiliary dysfunction characterized by portal inflammation (Demircan et al., 1998). In our lab, a study conducted on piglets given different doses of Al plus PN solution revealed that high doses of Al (1500 µg/kg/ day) and the PN solutions with Al content 37.8 µg/kg/day caused cholestasis (Li, 2005). At the cellular level, Al accumulation in the liver has been shown to cause ultrastructure changes. In a study conducted by Galle et al on six patients, the results showed that intracellular accumulation of Al caused damage to the hepatocytes. In addition, he reported that Al was deposited in the hepatocytes not in the Kupffer cells (Galle et al., 1987). In a study conducted on mice administered Al chloride orally, Al was shown to deposit in the lysosomes of hepatocytes (Kametani et al., 2006). Besides its effect on the morphological structure, Al loading affects the bile acid level in the serum.

Klein et al., (1989) showed that Al deposition in the liver cause increased serum bile acids concentration. Despite the relationship between Al loading and liver injury, the exact role of Al in the development of PNAC is unknown. More studies are needed to reveal the exact underlying mechanism of Al in causing PNAC.

2.7 Iron

Iron (Fe) is an important component of hemoglobin, myoglobin, and many enzymes in the body and thus an important micronutrient in the well-balanced human diet (Guyton, 2006). The total quantity of iron in the human body averages 4000 to 5000 mg, about 60-70% of which is in the form of hemoglobin and myoglobin, while 20-30% is stored in the liver, in the form of ferritin and hemosiderin. The remaining about 10% is in the form of the various heme compounds that promote intracellular oxidation, cytochromes and other iron containing enzymes. Humans ingest approximately 12-18 mg/day of dietary iron, of which only 1-2 mg is absorbed (Crichton et al., 2002). Maintaining cellular iron homeostasis is vital to the physiological body functions. In the healthy individual, the concentration of free cellular iron is monitored by iron regulatory proteins (IRPs) that regulate the transferrin and ferritin receptors. However, individuals with hemochromatosis, a hereditary disorder, or transfusional iron overload have difficulties in maintaining iron homeostasis. The disruption of iron homeostasis can cause serious health problems. Elevated concentrations of cellular iron can cause oxidative damage to the cell and are linked to the pathogenesis of various diseases such as cancer and neurodegenerative disorders (Adzersen et al., 2003; Deugnier, 2003; Toyokuni, 2002; Jang and Surh, 2002; Ng, 2004). Evidence suggests that iron overload has been shown to result in increased lipid peroxidation, DNA lesions, and apoptosis induced by reactive oxygen species (Bacon et al., 1983; Kell, 2009).

Several studies have suggested that Al can alter iron homeostasis (Ward et al., 2001; Contini et al., 2007). One of the proposed hypotheses is that Al can decrease ferritin synthesis and increase the expression of transferrin receptors. The disruption of the normal synthesis of transferrin receptors and ferritin create increased free iron levels in the cell, thus result in an increase of oxidative damage (Abreo et al., 1994; Yamanaka et al., 1999). Another suggested mechanism is that Al displaces iron from transferrin and ferritin (discussed in section 2.6.5). Parenteral nutrition solutions are contaminated with significant amounts of Al. Infants and children with gastrointestinal problems who need PN therapy are predisposed to disrupted cellular iron homeostasis in the liver, which results in oxidative damage and liver injury.

2.7.1 Intestinal absorption of iron

There are two main forms of dietary iron, which are absorbed by the digestive tract; heme iron from meats, and non-heme iron from plant and dairy products. Heme iron is more efficiently absorbed than non-heme iron. However, heme iron comprises only about 10% to 15% of daily iron intake (Carpenter and Mahoney, 1992). In the duodenum, the main site of dietary iron absorption, heme and non-heme iron pass from the intestinal lumen to the enterocyte across the brush border by different pathways (Siah et al., 2005). Heme iron is more readily absorbed and enters the enterocyte as an intact metalloporphyrin (Uzel and Conrad, 1998). Heme iron is taken up into the enterocyte via a heme receptor, heme carrier protein-1 (HCP1), on the brush border of intestinal cells (Shayeghi et al., 2005). Once in the enterocyte, heme is broken down into free iron and biliverdin by heme oxygenase that is found on the endoplasmic reticulum surface (Raffin et al., 1974). Iron liberated from heme then enters the intracellular or 'labile' iron pool. For non-heme iron, absorption occurs as a multi-step process. Non-heme iron, which occurs mainly in the ferric state (Fe III), is first reduced to ferrous (Fe II) in the intestinal lumen

by a ferrireductase at the apical surface of the brush border. Ferrous iron (Fe II) is then transported across the cellular membrane by divalent metal transporter 1 (DMT-1) into the enterocytes of the villus tips of the duodenum, which is the site of the major iron absorption (Gunshin et al., 1997). Divalent metal transporter 1, the only known Fe transporter in the intestine, also transports other divalent metals including zinc, manganese, cobalt, copper, cadmium, nickel and lead (Gunshin et al., 2005). Once in the cell, the Fe(II) is either stored as ferritin, incorporated into iron regulatory proteins (IRP), or is transported to the basolateral membrane and excreted by the cell via an iron transporter protein, known as iron-regulated protein 1 (IREG1), into the circulation. The released iron is oxidized to the ferric ion by hephaestin and binds apo-transferrin to form Fe (III)-transferrin complex (Vulpe et al., 1999). Transferrin is the major iron transporter in the body and can bind 2 molecules of the oxidized form of iron (Rouault and Klausner, 1997).

2.7.2 Hepatic uptake of iron

Transferrin (Tf) is responsible for the majority of cellular iron delivery within the body. In the hepatic cells, diferric-Tf attaches to the transferrin receptor (TfR) on the surface of the cell membrane and the Tf:TfR complex is internalized by receptor-mediated endocytosis via coated pits (Richardson and Ponka, 1997). Once within the cell, the coated-vesicles move within the cytoplasm until they fuse with trans-reticular Golgi elements to form an endosome (McClelland et al., 1984). The iron released from Tf is translocated from the endosome to the cytosol by the DMT1. The apoTf:TfR (transferrin without iron) complex is then transported back to the cell surface. The iron released in the cytosol is either stored as ferritin or incorporated into mitochondria for the synthesis of heme protein (Beard et al., 1996).

Non transferrin-bound iron (NTBI) is defined as iron bound to other molecules than

transferrin such as citrate, ascorbic acid, or nitrilotriacetic acid. Irrespective of the form of NTBI presented to cells, NTBI taken up first by binding to the cell surface where iron dissociates from its ligand; the dissociation possibly involves cell surface reduction by a ferrireductase. Iron then delivered into the cell by a transporter (Graham et al., 1998; Trinder and Morgan, 1998). The identity of the transporter for the NTBI uptake in the hepatocyte is unknown yet.

2.7.3 Cellular iron homeostasis

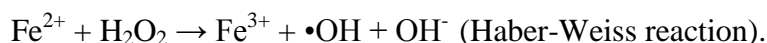
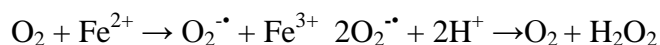
The uptake, sequestration, and export of iron must be properly regulated

c. The regulation of intracellular iron concentration is through the control of iron uptake, and is influenced by iron storage capacity. As the intracellular functional iron concentration drops, iron is mobilized from ferritin stores and intracellular ferritin levels are reduced, while transferrin receptor synthesis is activated and the transferrin receptor concentration on the cell surface increases. Similarly, as intracellular iron concentrations increases, ferritin molecules are synthesized and the number of transferrin receptors on the cell surface is reduced. This process is regulated by two cytosolic IRE (iron responsive elements) - binding proteins known as iron regulatory proteins; IRP-1 and IRP-2 (Crichton and Ward, 1995). They act as iron sensors, essentially by existing in two different conformations. When the supply of iron is increased, the IRP-1 is post-transcriptionally inactivated and IRP-2 degraded, both IRP-1 and IRP-2 become unavailable for IRE binding, allowing transferrin mRNAs degradation and ferritin mRNA translation. (Iwai et al., 1995; Hentze and Kuhn, 1996). When iron levels are low, the IRPs are activated and bind to IRE on both ferritin and transferrin mRNAs (Meneghini, 1997). This binding stabilizes the transferrin mRNA and inhibits translation of the ferritin mRNA. Hence, when iron levels are low there is an increase in the synthesis of transferrin and a decrease in ferritin synthesis. Conversely, when there is an adequate supply of iron, IRP binding is

deactivated and the opposite result is observed.

2.7.4 Iron and oxidative damage

Oxidative damage or stress has been defined as an imbalance between the pro-oxidant/antioxidant steady state in the cell. Free radicals are highly reactive chemical species that maintain one or more unpaired electrons (Halliwell, 1990; Chakravarti et al., 1991). A compound can become a free radical by either gaining or losing an electron. Although iron is important for many normal cellular activities, excess causes devastating toxic effects. Ferrous iron (Fe^{2+}) can react with hydrogen peroxide to produce ferric iron (Fe^{3+}) leading to the formation of hydroxyl radicals; $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \bullet\text{OH} + \text{OH}^-$ (Fenton reaction). Iron can also react with molecular oxygen to yield a variety of toxic chemicals, such as superoxide anions ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet\text{OH}$);



These reactive oxygen species (ROS) are believed to play important pathogenic roles in a variety of diseases with excess iron such as Alzheimer's disease and Parkinson's disease (Dexter, 1989; Good et al., 1992; Mattson, 1995; Markesbery, 1997). Free radical formation can promote lipid peroxidation, DNA strand breaks, degradation of biomolecules, and eventually cause cell damage (Halliwell and Gutteridge, 1984; Watson et al., 1984; Komara et al., 1986; Siesjo, 1988). Therefore, biological systems have evolved specialized iron-transport and management systems to maintain iron in a soluble nontoxic form and developed mechanisms to prevent increase of the iron-pool while maintaining sufficient levels for metabolic use. However, these homeostatic mechanisms can get dysregulated and cause iron overload or iron deficiency.

2.7.5 Aluminum, iron interaction, and oxidative damage

Al is a non-redox trivalent cation, which is capable of increasing the cellular oxidative environment by potentiating the pro-oxidant properties of transition metals such as iron and copper (Bjertness et al., 1996). Under normal physiological conditions, when the cell needs iron, transferrin receptor (TfR) production increases allowing for more iron to be brought into the cell and the storage protein ferritin decreases enabling more iron to reach the respiratory chain and other iron requiring systems. When the cells have enough iron, TfR decreases and the levels of ferritin increase thus allowing iron to be stored in the ferritin which prevents iron-mediated oxidative stress (Aisen et al., 2001). However, when the cell is loaded with Al, the cellular accumulation of Al can alter iron metabolism and induce peroxidative injury. Since Al and iron bind to transferrin (Tf) and ferritin, Al could disrupt cellular pathways of iron and thus indirectly increasing the intracellular concentration of free iron. A number of studies conducted on a variety of cultured cell lines have shown that Al loading results in abnormal iron accumulation and compartmentalization (Abreo et al., 1994; Abreo et al., 1999; Yamanaka et al., 1999). In friend erythroleukaemia cells (FEC), Al loading has been shown to disrupt iron metabolism by increasing cellular iron content associated with increased compartmentalization of iron in the mitochondria and nuclei, decreased ferritin content, and decreased uptake of iron by ferritin (Abreo et al., 1994) which lead to an increased iron to ferritin ratio in the cell. This increase in iron to ferritin ratio would alter the normal compartmentalization of cellular iron, which has been shown to be associated with increased membrane lipid peroxidation (Bacon and Britton, 1989; Abreo et al., 1994). It is not clear how Al causes reduction in the ferritin concentrations in the tissues. However, suggested mechanisms include inhibition of translational regulation of ferritin synthesis either by sequestration of iron in a cellular compartment that is not accessible to iron regulatory protein (IRP) or by competing with iron for binding sites on IRP. Either mechanism

could result in less iron binding to the IRP, thereby inhibiting the synthesis of ferritin (Abreo and Glass, 1993). The decrease in synthesis of ferritin is associated with an increase of iron concentrations. This increase of iron ratio to ferritin would imply that the normal compartmentalization of cellular iron is altered, which is associated with increased membrane lipid peroxidation.

Aluminum can facilitate lipid peroxidation in the presence of free iron under acidic conditions *in vitro* (Verstraeten and Oteiza., 1995; Ohyashiki et al., 1996). Increased malondialdehyde (MDA), a by-product of lipid peroxidation, has been shown in Al-loaded mouse hepatocytes (Abreo et al., 1990). Furthermore, in rat liver microsomal fractions, Al ions have been shown to accelerate iron induced lipid peroxidation (Quinlan et al., 1988). In studies focused on the correlation between Al accumulation and Alzheimer disease (AD), Al has been shown to stimulate lipid peroxidation in the presence of ferrous iron (Fe II) in brain membranes (Oteiza, 1993). Several studies clearly established that the presence of Al in the cell plays a role in causing oxidative damage via disrupting normal compartmentalization of iron within the cell, however; no work looked at Al and iron interaction in parenteral nutrition infused animals, and the mechanisms involved in causing liver injury. Knowing that would shed the light on the role of Al and iron in causing liver damage in parenteral nutrition associated cholestasis.

2.8 Biomarkers of oxidative stress

Oxidative stress has been proposed as a significant factor in many diseases. However, definitive evidence for its association with diseases has often been lacking due, in part to the lack of biomarkers and/or reliable methods available to assess oxidative stress levels (Mak and Newton, 2001). For instance, previous research has proposed reactive oxygen species (ROS) to be short-lived and highly reactive making the accuracy of their *in vivo* measurement

questionable. A biomarker for oxidative stress is a biological molecule that changes when reactive oxygen species and/or free radicals change, and can be objectively measured and evaluated (Offord et al., 2000). Biomarkers have been shown to yield important information on the nature of radical damage and antioxidant action *in vivo*, particularly regarding the nature of pro-oxidant effects, compartmentalization, and bioavailability (Bartosz and Bartosz, 1999).

There are several methods to assess oxidative stress *in vivo* and *in vitro* including measuring products of oxidative stress. Such methods include DNA oxidation (Mendez et al., 2004) and lipid peroxidation (Hsiang et al., 1997), or protein modification (Chirino et al., 2006); measurement of the reducing capacity of tissue or cell (Shohami et al., 1999), and trapping the superoxide radicals using electron spin resonance trapping reagents (Awasthi et al., 1997). However, measuring lipid peroxidation by-products, DNA oxidation markers, and protein carbonyls are currently the most commonly used methods.

2.8.1 Lipid peroxidation

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and used as an indicator of oxidative stress in cells and tissues (Armstrong and Browne, 1994; Yagi, 1998). Masuda and Yamamori (1991) reported increased concentrations of lipid peroxidation products in some diseases associated with oxidative stress. For example, peroxidation appears to be important in diseases such as atherosclerosis (Durak et al., 2000), tissue injury caused by ischemic or traumatic brain damage (Peker et al., 2004), and hepatic damage induced by toxic substances such as trichlorobromomethane (Slater, 1984), chloroform (Ekstrom and Hogberg, 1980), and halothane (Tomasi et al, 1983).

This oxidative degradation process results from the production of free radical reactions primarily involving membrane polyunsaturated fatty acids (PUFA) (Slater, 1984; Poli et al,

1987). Highly reactive free radicals are capable of abstracting hydrogen atoms from polyunsaturated fatty acids on phospholipid membranes, resulting in the formation of a lipid radical (PUFA•). Reaction with oxygen yields the corresponding peroxy radical (PUFAOO•) and chain propagation, leading ultimately to degradation of the lipid to a range of products including aldehydes, ethane and pentane. Malondialdehyde (MDA), an aldehyde, is one of the most common lipid peroxidation products that are predictive of oxidative stress (Griffiths et al., 2002). It is a physiologic ketoaldehyde produced mainly by peroxidative decomposition of unsaturated lipids as a by-product of arachidonic acid metabolism. Malondialdehyde can be measured using thiobarbituric acid-reactive substances (TBARS) assay, a well-established method for screening and monitoring lipid peroxidation (Armstrong and Browne, 1994; Yagi, 1998). The method based on the reaction of TBA with malondialdehyde (MDA) and other reactive aldehydes. Modifications of the TBARS assay by many researchers have been used to evaluate several types of samples including human and animal tissues and fluids, drugs, and foods (Ohkawa et al, 1979; Richard et al, 1992; Draper et al, 1993; Scoccia et al, 2001; Dawn-Linsley et al, 2005). Even though there remains controversy in the literature regarding the specificity of TBARS toward compounds other than MDA (Janero, 1990), it remains the most widely employed assay to determine lipid peroxidation (Armstrong and Browne, 1994; Esterbauer, 1996; de Zwart et al, 1999).

Another product of lipid peroxidation used extensively as a sensitive and specific marker of oxidative stress is F2-isoprostane (Morrow et al., 1990). Isoprostanes are oxidation products of arachidonic acid. A study by Roberts and Morrow (1997) reported that there are two separate routes of isoprostane formation, endoperoxide and a dioxethane. During isoprostane formation, 8-iso-IPF2- α is used as a marker for oxidative stress (Morrow and Roberts, 1999). Additionally,

in plasma, studies have reported isoprostanes to have a short half-life of approximately 18 minutes and to be excreted rapidly. To maintain steady state, it must be formed constantly in biological substances (Morrow and Roberts, 1999). The disadvantage associated with measuring isoprostanes in plasma is that it is not possible to measure them over a period of time since the half-life is short. Conjugated dienes have also been proposed to be a product of *in vitro* lipid peroxidation. Conjugated dienes are primary products of the breakdown of fatty acids. However, a disadvantage associated with the diene assay is that many other biological substances are absorbed in the same UV region (Corongiu et al., 1986), thus making conjugated diene assay not appropriate for determination of lipid peroxidation *in vivo*.

2.8.2 Amino acid oxidation

Lipid peroxidation consequences including amino acid oxidation, denaturation of proteins, and loss of function to the proteins are also used as indicators. For the analysis of oxidation products, proteins, urine, plasma, cell, nucleus, mitochondria, and cytoplasm can be used. In a study by Griffiths et al. (2002), it was shown that modifications to histidine and lysine in low-density lipoproteins lead to an alteration in the receptor lipoproteins. Protein oxidation stimulates lead to new functional group formation that includes hydroxyls and carbonyls (Dean et al., 1997). Carbonyls have emerged as excellent biomarkers for protein oxidation (Berlett and Stadtman, 1997). They are generated in response to oxidizing stimuli including alkoxy and peroxy radicals. As a biomarker, carbonyls measured by either immunodetection using ELISA or Western blot. Protein carbonyls are a generic marker of oxidation (Pompella et al., 1996). In a review by Chevion et al. (2000), alterations in protein carbonyls were shown to be linked with aging. Previous research has described protein thiols, aliphatic amino acids, oxidized tryptophan and tyrosine as biomarkers for oxidative stress (Robinson et al., 1998; Griffiths et al., 1992, and

Shigenaga, 1999). Semi-quantitative analysis of thiols immunohistochemically by Wardman and Von Sonntag (1995) showed a correlation with tumor growth (Wardman and Von Sonntag, 1995). Regardless of the changes in protein structures and diseases, studies have shown that supplementation of diets with antioxidants can reverse impact on some of those modifications in protein carbonyls. Supplementation of a rat diet with flavonoid rutin for 18 days caused a reduction in protein carbonyl content (Funabiki et al., 1999). Srigiridhar and Nair (2000) supplemented the diet of rats with α -tocopherol or a combination of α -tocopherol (40 mg/day) and ascorbic acid (24 mg/day) for 15 days and showed that protection against protein carbonyl formation in iron-deficient rats during iron repletion increased. Tocotrienols reduced the protein carbonyl levels in the aging nematode, *Caenorhabditis elegans* (Adachi and Ishii, 2000). These limited studies are consistent in demonstrating a protective effect against plasma protein oxidation through dietary intervention.

2.9 The piglet as a model for PNAC

Several animal models of PN including mice, rats, rabbits, and neonatal piglets have been used to study liver disorders of PN-induced liver injury. An ideal animal model of human infant should produce viable neonates with maturation characteristics similar to human infant, and have a body size that allows comparative monitoring, blood sampling, clinical interventions, and easily to handle. Also, the growth of piglet is very rapid during the neonatal period which can be viewed as an accelerated model of postnatal growth and development (Sangild, 2006).

Numerous studies have used both adult and neonatal piglets to study liver dysfunction (Klein et al., 1987; Truskett et al, 1987; Omland and Mathisen, 1991). The newborn piglet have been used as a valid model for studying neonatal metabolism in orally fed animals (Innis, 1993; Moughan et al., 1992), and in intravenously fed animals (Wykes et al., 1993; Shulman, 1993;

Dureksen et al., 1996; Van-Aerde et al., 1999; Bertolo et al., 1999). Neonatal piglet is an ideal animal for modeling liver function and metabolism as it has a similar hepatic anatomy; gastrointestinal physiology, and metabolism to human neonate (Yuan et al., 1996). Similar clinical complications to those observed in human infants on PN have been shown in piglets (Tumbleson, 1986; Schantz et al., 1996). The liver injury observed in piglets receiving PN resembled that seen in human neonates (Zambrano et al., 2004; Wang et al., 2006). In addition, the piglet model has been used to screen novel components for parenteral nutrition formulas in clinical practice (Hata et al., 1989; Shu et al., 1991; Burrin et al., 2000; Burrin et al., 2003; Wykes et al., 1993; Loff et al., 1998; Moran et al., 2005; Cai et al., 2006).

The newborn piglet provides the opportunity to assess the relative importance of putative growth-regulatory and survival factors in a clinically relevant context. The observed outcomes in neonatal piglets that develop PN-induced liver disease would likely more relevant to human infants than outcomes demonstrated by experiments in other animal models.

2.10 Biological imaging techniques

2.10.1 Overview

Biological imaging is a demanding field, which requires several important factors to be balanced in order to achieve the best result. Generally, an imaging technique for biological samples should achieve high spatial resolution to reveal information at the cellular and sub-cellular level, require minimal sample preparation to examine samples as close as possible to their natural state, and use a radiation dose that is tolerable to minimize sample alterations.

Although, light and electron microscopy techniques have been extensively used to identify the structural changes in the liver tissues of rats resulting from infusion of aluminum (Klein et al., 1988; Dermican et al., 1998), there are number of limitations associated with these two techniques. For instance, the low spatial resolution (hundreds of nanometers) by light

microscopy limits identification of sub-cellular changes and extensive sample preparation method for electron microscopy alters the sample composition. A technique that can provide high spatial resolution like electron microscopy (~ 2 nm) with minimum sample preparation for light microscopy is highly desirable for this study.

One such technique that provides the advantages of both electron and light microscopy is the X-ray microscopy. The Scanning Transmission X-ray Microscopy (STXM) is a synchrotron based technique and has the potential to reveal ultrastructure of intact and hydrated cells at high spatial resolution (~ 30 nm) (Jacobson, 1999). The spectroscopy associated with microscopy can be used to identify and reveal the different states of different elements present in the sample. However, other conventional techniques such as Raman and confocal microscopy also have been used extensively in biological science for morphological, elemental and chemical imaging of cells. In the following sections, the use of light, Raman, Confocal and electron microscopy and comparative advantages of STXM are highlighted with examples related to cholestasis.

2.10.2 Light Microscopy

The light microscope is a critical tool in the study of cellular structural and functional characterization related to pathology. Improvements in lens production for higher magnification with the parallel development of sample preparation such as fixing, sectioning and staining of cells and internal tissues have enabled biologists to use it for wide range of applications, ranging from investigations of bacterial cells to human tissues. Studies of biological structures and processes on both fixed and live specimens have led light microscopy to be a vital tool for biologists. The light microscope creates a magnified and detailed image of objects or specimens invisible to naked eye. In light microscopy, objects are enlarged or magnified with a convex lens that bends light rays by refraction. The visibility of the magnified object depends on contrast

and resolution. In general, the contrast or differences in light intensity between an object and its background or surroundings make the object distinct. For colorless or less contrast specimens, as in the case of most biological material, contrast is achieved in different ways. The object itself or selected portions of a specimen may be stained, thus enhancing the amplitude of certain light waves passing through the stained areas. However, this usually requires the fixation and staining of cells. Such stained specimens are typically observed using bright-field microscopy. Despite these developments, the detail at the sub-cellular level is very limited in light microscopy. The maximum spatial resolution achieved so far is about 200 nm due to the long wavelengths of visible light used in light microscopy (Fernandez-Suarez and Ting, 2008).

2.10.2.1 Light Microscopy and cholestatic features

The histological features of the cholestatic liver are very important for the diagnosis of cholestasis. The common features of cholestasis are bile thrombi in the canaliculi (canalicular cholestasis), periportal inflammation, bile duct proliferation and portal fibrosis (Dermican et al., 1998; Btaiche and Khalidi, 2002). Fibrosis, portal inflammation, bile duct proliferation, and hydropic degeneration are the most prominent histological features of PN-related liver damage (Quigley et al., 1993; Merritt, 1986; Benjamin, 1981; Dahms and Halpin, 1981; Hodes et al., 1982; Moss et al., 1993). Light microscopy has been used extensively to detect (Figure 2.3) and study the histological features of cholestasis (Postuma and Trevenen, 1979; Shu et al., 1991; Loff et al., 1998; Cai et al., 2006; Wang, 2006). Even though light microscopy has been an important tool used to detect many structural changes in the cholestatic liver tissues, in some studies, light microscopy could not detect some structural changes such as loss of bile canalicular microvilli, and swollen mitochondria, as well as some apoptotic changes such as karyoklasis (Shu et al., 1991; Cai et al., 2006).

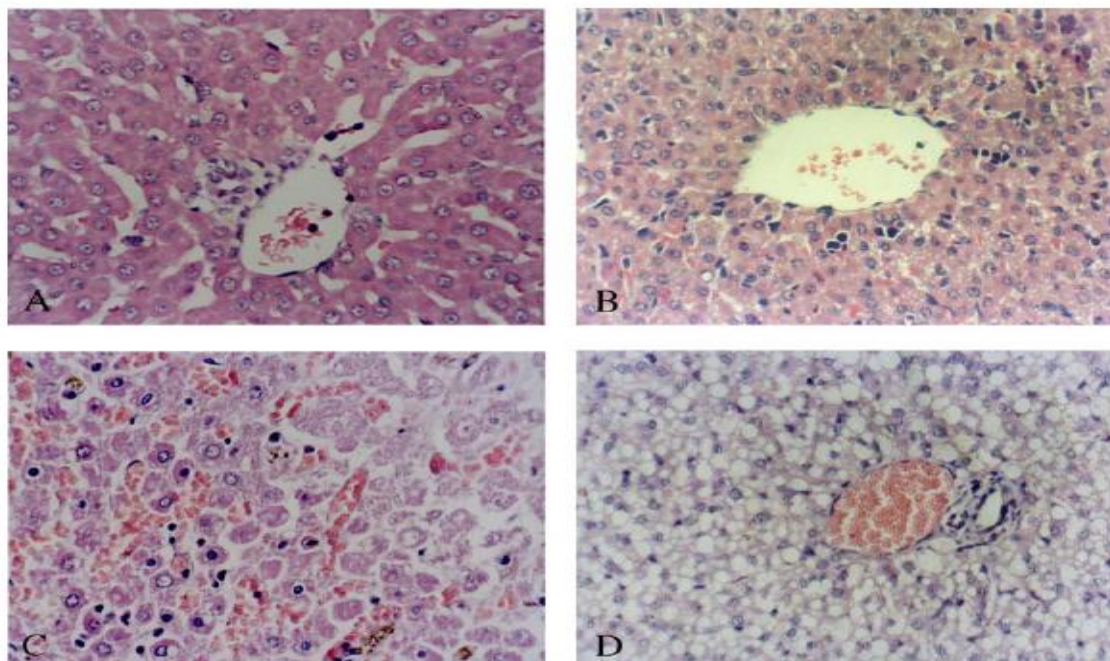


Figure 2.3. Light micrograph of liver tissues;

A) Control group, no degeneration or cholestasis; B) parenteral nutrition for 7 days, mild inflammatory infiltration and hydropic degeneration; C) PN for 10 days, hepatic intracellular cholestasis; D) PN for 10 days, diffuse fatty degeneration.

(Source: Cai et al., 2006).

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2.10.3. Raman microscopy

Raman microscopy (RM) is a vibrational spectroscopic technique capable of obtaining sensitive measurements of molecular composition, structure, and dynamics of inorganic compounds, organic molecules, and minerals. It is fast becoming one of the most powerful analytical techniques that can be applied to many areas of research including materials science, forensic research investigation, biological, biomedical materials (Yoshikawa and Nagai, 2001; Nishimura and Tsuboi, 1986; Paipetis et al, 1996; Schaeberle et al., 2001; Hodges and Akhavan, 1990; Cutmore and Skett, 1993). The main advantages are that the technique is non-destructive and there is minimal sample preparation. The use of Raman spectroscopy in the biological

sciences has become incredibly widespread due to its advantages. Some of the successful application of RM includes single-cell analysis (Puppels et al., 1990), characterization of the molecular composition of bacteria and other medically relevant microorganisms (Naumann et al., 1995; Maquelin et al., 2002). The technique has also been applied to single human tumor cells, and has been used successfully to map the distribution of protein and nucleic acids within the cytoplasm and nucleus (Krafft et al., 2003; Matthaus et al., 2006). Many investigators have used RM to characterize the spectral differences between normal and cancerous cells and tissues, or to distinguish between different types of tumor cells (Nijissen et al., 2002; Choi et al., 2005; Crow et al., 2005; Short et al., 2006; Jong et al., 2006). RM has also been used to investigate the spectral differences between living and dead tumor cells (Notingher et al., 2003) and to monitor the spectral changes of tumor cells undergoing cell death via apoptosis (Verrier et al., 2004). Moreover, RM has been used to differentiate between the AI-treated and untreated brain tissue samples (Abubaker et al., 2002).

Raman spectroscopy is increasingly used to provide chemical structure information on tissue specimens. However, because of the low spatial resolution, details of the histologic structure at the sub-cellular level are very limited.

2.10.4 Confocal microscopy

Confocal microscopy is an imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane. In confocal microscopy (CM), illumination of the sample is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen. The resulting fluorescence transmission is collected by the objective and sent to the detector in the same manner as the wide field illumination mode.

Confocal microscopy offers several advantages over conventional optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane, and the capability to collect serial optical sections from thick specimens. In biological and biomedical application, the importance of confocal microscopy as non-destructive and sensitive technique made it widely used in a broad range of applications including neuroanatomy and neurophysiology, stem cell research, bioluminescent protein and photobleaching studies, DNA hybridization and morphological studies of a wide variety of cells and tissues (Halbhuber and Konig, 2003).

One of the most widely used confocal microscopy techniques in biological application is the laser scanning confocal microscopy (LSCM). This technique relies heavily on fluorescence as an imaging mode, primarily due to the high degree of sensitivity afforded by the technique coupled with the ability to specifically target structural components and dynamic processes in chemically fixed as well as living cells and tissues. Many fluorescent probes designed to bind with a biological macromolecule (for example, a protein or nucleic acid) or to localize within a specific structural region, such as the cytoskeleton, mitochondria, Golgi apparatus, endoplasmic reticulum, and nucleus (Haugland, 2005). Other probes are employed to monitor dynamic processes and localized environmental variables, including concentrations of inorganic metallic ions, pH, reactive oxygen species, and membrane potential (Lemasters et al., 1999).

Fluorescent dyes are also useful in monitoring cellular integrity (live versus dead and apoptosis), endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction, and enzymatic activity (Johnson, 1998). In addition, fluorescent probes have been widely applied to genetic mapping and chromosomes analyses in the field of molecular genetics. For elemental mapping, in a study conducted by Uchiumi et al., (1998), laser scanning confocal

microscopy was used to detect Al in the liver and tibia of normal and renal failure Wistar rats administered intraperitoneally with 1 mg injections of Al potassium sulfate three times for a period of 3 or 6 months. However, using lumogallion as stain, reacts with Al to form fluorescent complex, this experiment has enabled the investigator to detect Al in tissues containing $> 9 \mu\text{g/g}$ of Al but not $< 6.5 \mu\text{g/g}$ of Al (Uchiumi et al., 1998).

In previous years, some researchers employed confocal microscopy to investigate the mechanisms underlying the development of some hepatobiliary disorders including cholestatic diseases. Kojimi et al., (2007) successfully used confocal microscopy to investigate the localization and expression of multidrug resistance protein 2 (MRP2), and radixin, a cross- linker between actin filaments and membrane proteins in various cholestatic liver diseases. Also, Li et al., (2009) used this technique to investigate the effect of hepatic ischemia-reperfusion on the bile canalicular F-actin microfilaments, main component in the structure of bile canalicular microvilli, in rats.

The use of confocal microscopy, especially the laser scanning confocal microscopy is rapidly growing and becoming an invaluable tool for a wide range of investigations in the biological and medical sciences. However, because of the low spatial resolution compared to the electron microscopy, the use of this technique is still limited as tool for the histological observation in cells and tissues.

2.10.5 Transmission electron microscopy (TEM)

The transmission electron microscope (TEM) uses electrons as light source to study objects at very high spatial resolution. TEM provides the means to go beyond the magnification and resolution limits of light microscopes, allowing for magnification of up to 100,000 X and resolution in the nanometer range. However, the weakness of electron penetration requires

sample thicknesses considerably less than 1 μm (Kirz et al., 1995). Sectioning samples into thin sections can cause structural artifacts or distortion. The other challenge is the low contrast of biological materials which requires staining samples with heavy metal salts that bind to specific intracellular organelles to differentiate them which can be another source of artifact. There is well-documented evidence for the loss of cellular material especially during chemical fixation and subsequent dehydration of biological specimens (Coetzee and van der Merwe, 1984, 1989; Mersey and McCully, 1978; Salema and Brandao, 1973). More recent techniques such as cryo-fixation avoid the need for some of these sample preparation methods like chemical fixation but can cause structural disorganization from freezing damage.

2.10.5.1 Transmission electron microscopy and cholestatic features

Similar to light microscopy, transmission electron microscopy has been used extensively to study the subcellular structural changes of cells in tissues. In light microscopy, cell membranes and a few internal organelles could be visualized, but the TEM image easily reveals much more structures in the organelles (Figure 2.4; Wang et al., 2006). In detecting the cholestatic features in liver tissues, due to its high spatial resolution, electron microscopy has the advantage of detecting some cholestatic features that could not be detected using light microscopy such as bile plugs, loss of canalicular microvilli, apoptotic changes (Fig 2.5; Cai et al., 2006), and swollen mitochondria (Kametani, 2002).

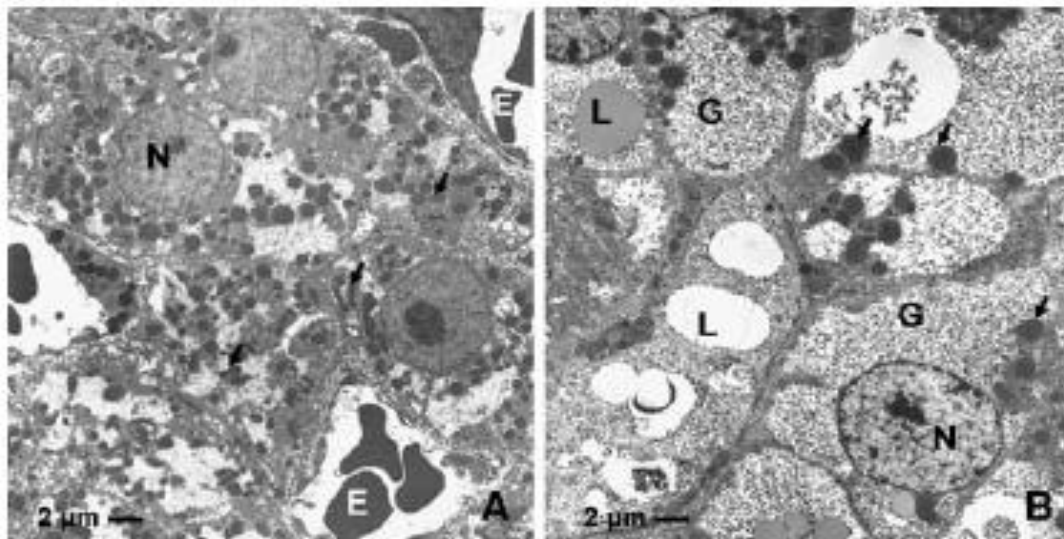


Figure 2.4. Transmission electron micrographs showing structure in piglet livers of EN (enteral nutrition) group (A) and PN (parenteral nutrition) group (B).

In EN; nucleus (N), and cytoplasm in hepatocytes and presence of erythrocytes (E); arrows indicate mitochondria. In PN liver specimens morphological characteristics of fatty liver with swollen and rounded mitochondria and accumulation of the lipid droplets (L) and granules of glycogen (G) in cytoplasm are evident.

(Source: Wang et al., 2006).

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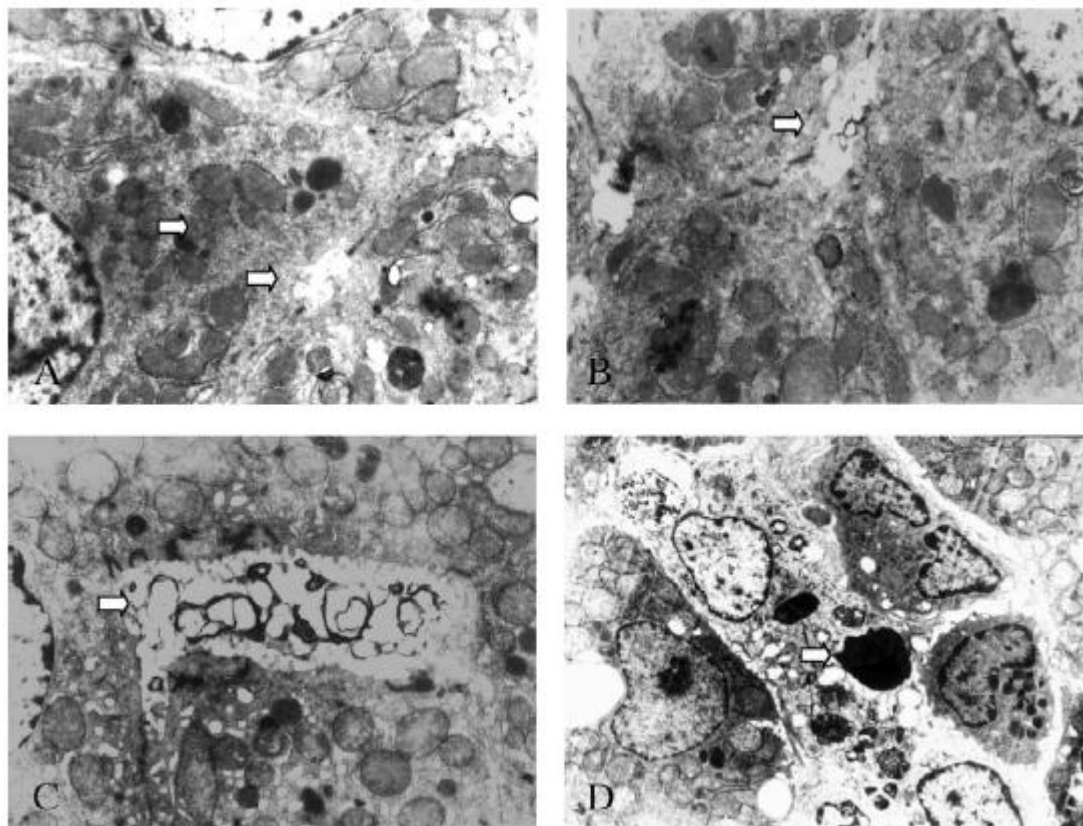


Figure 2.5. Electron microscopy of rabbit livers;

A) Control group, normal mitochondria and micro bile duct (see arrow); B) PN-3 group, enlarged micro bile duct (see arrow). C) PN-10 group, high electron density laminar deposition in enlarged micro bile duct (see arrow); D) PN-10 group, apoptosis body in hepatic sinusoid (see arrow). (Source: Cai et al., 2006)

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2.10.6 Elemental mapping in biological tissues

The mapping of element distribution in thin sections of biological tissues is a challenging task. Exploring the subcellular metal deposition in the tissues is a key to the understanding of many metal-involving disorder mechanisms in biological tissues. Conventional imaging techniques used to gain knowledge about the elemental distribution in tissues or cellular or sub-cellular level, usually lack sensitivity to detect trace concentrations of elements in the biological

tissues. Recently, imaging the distribution of trace elements in cells (Ortega et al., 2004) and tissue sections at the cellular and subcellular compartments is becoming possible using synchrotron radiation X-ray techniques. Moreover, the efficient use of synchrotron beams will provide information about oxidation state and metal-site structures at subcellular level (Ortega et al., 2005).

2.10.6.1 Aluminum mapping in biological tissues

Al is abundantly found in the earth and dissolved into acidic rains thus spreading into the earth's soil and water, which can be absorbed into living bodies of both the animals and plants and can be accumulated in cells and tissues (Martin, 1986). The toxicity of Al accumulation in a living body was shown experimentally in plants such as in wheat (Delhaize et al., 1993) and in several organs such as the livers, kidneys and brains of experimental animals (Spencer et al., 1995; Wilhelm et al., 1990; Yokel, 2000). Many imaging techniques were employed to look at the deposition of Al in the biological materials. Some of these techniques and their use in mapping Al will be discussed subsequently.

2.10.6.1.1 Light microscopy

There are few studies where light microscopy employed to detect the deposition of Al in biological tissues. In a study conducted on Wistar rats received five intraperitoneal injections at dose of 50 mg/kg, Daimon et al., used light microscopy to locate the deposition of Al in liver tissues stained with aluminon and he reported that Al was localized in the cytoplasm of Kupffer cells (Daimon et al., 1999). In another study, Al deposits were detected using light microscopy in the Kupffer cells and macrophages of Wistar rat's liver tissues stained with aluminon. The rats were given intraperitoneally daily injections of 50 mg Al for 7 days (Bogdanovic et al., 2008).

2.10.6.1.2 Energy dispersive microanalysis (EDX)

X-ray microanalysis is a useful method to qualify and quantify basic elements in biological specimens. The term microanalysis was used to mean chemical analysis of very small amounts of elements in ultrathin sections in situ by X-rays using either transmission (TEM) or scanning electron microscope (SEM) equipped with X-ray analyzers (Chandler and Battersby, 1976). With scanning electron microscopy, one can observe and analyze only the surface of tissue blocks whereas using transmission electron microscopy, one can observe and analyze the inside of the tissues. In terms of X-ray analyzer, currently there are two types of X-ray analyzers available: wave dispersive X-ray analyzer (WDX) and energy dispersive X-ray analyzer (EDX). EDX has the potential to analyze all the elements in the sample at a time, whereas, WDX can only analyze one element at once. Thus, transmission electron microscopes equipped with EDX for biological specimens would have the potential to analyze all the elements with atomic number 11 (sodium) to atomic number 92 (uranium) in the sample at once. The aim of application of X-ray microanalysis to biological specimens is to detect various elements localized in the cells and tissues and to determine the kind of elements detected in situ, and further to quantify the contents of the elements in the specific cells, cell organelles and tissues which contain the elements in relation to their localizations.

2.10.6.1.2.1 Energy dispersive microanalysis (EDX) and aluminum mapping

Many researchers have explored the distribution of Al in biological tissues using energy dispersive microanalysis (EDX). Spencer et al. (1995) measured Al content in the livers and kidneys of rats at the cellular and subcellular level after intravenous administration of Al citrate and Al chloride. They reported that EDX could analyze the Al distribution in the cell organelles.

They determined that Al was distributed in all organelles analyzed such as cytoplasm, mitochondria, endoplasmic reticulum and nucleus of the liver cells (Spencer et al., 1995). In another study aimed to determine the best conditions of EDX to detect Al deposition in mouse tissues, several adult mice at four weeks of age were administered intraperitoneally and orally with 2% Al chloride for 2 weeks. The results suggested that the highest peak of Al was obtained at 300 kV when 1.0 μm thick sections were used. Using TEM-EDX, Kametani et al., (2002) reported that Al concentration was found in the lysosomes of hepatocytes in the liver tissues of mice administered orally with Al in drinking water for 3-17 weeks (Figures 2.6 & 2.7).

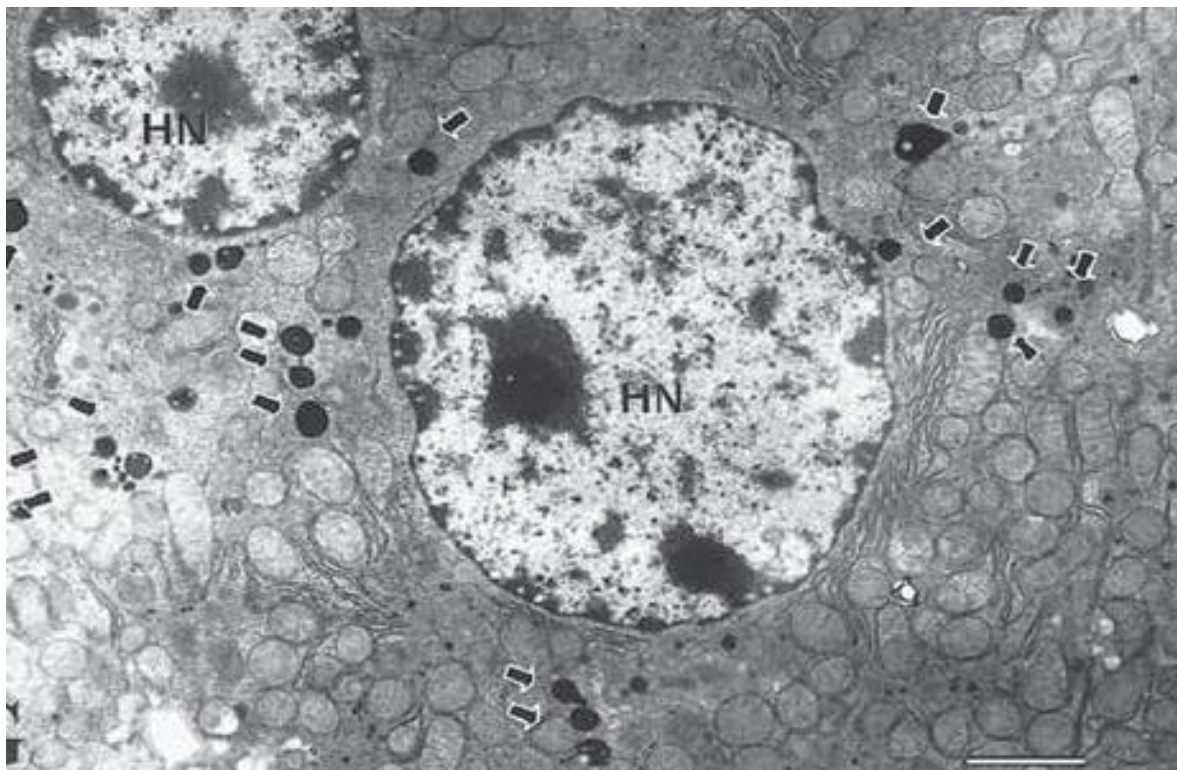


Figure 2.6. TEM micrograph for the hepatocytes of mouse administered with aluminum.

The high electron density (arrow) represents aluminum deposition in the lysosomes of mouse hepatocytes.

(Source: Kametani et al., 2002)

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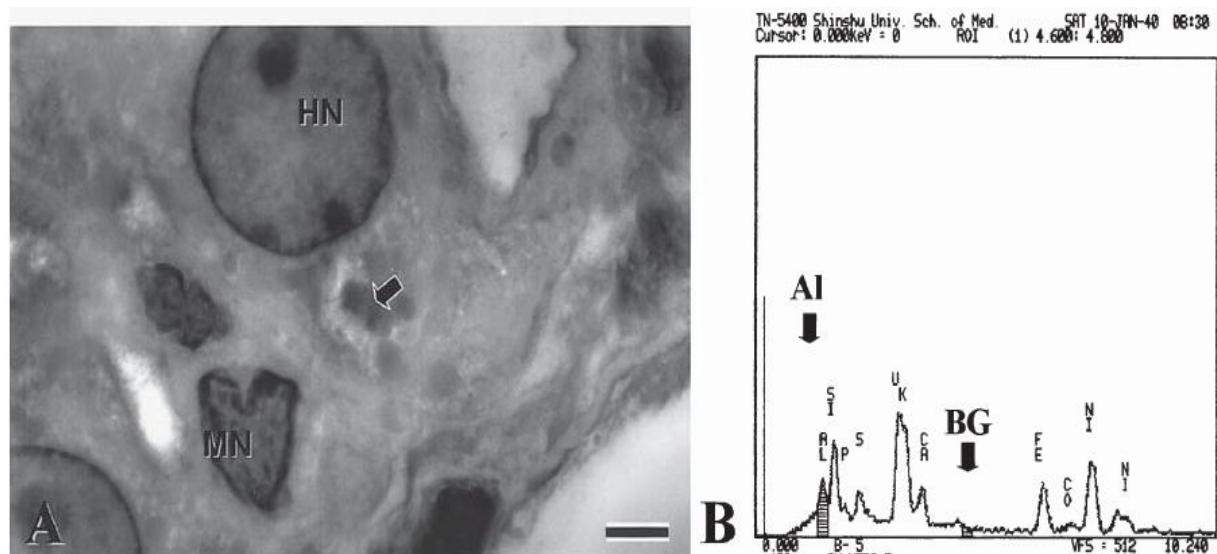


Figure 2.7. Electron micrograph of transmission electron microscopy showing aluminum deposited in the lysosomes of mouse

(A) Lysosomes (*arrow*) in a hepatocyte (HN) found in the liver of a mouse.

(B) EDX spectrum obtained from the electron-dense lysosomes (*arrow* in A). Al; aluminum, BG: background.

(Source: Kametani et al., 2002)

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2.10.7 Scanning transmission x-ray microscopy (STXM)

Scanning transmission x-ray microscope (STXM), shown on Fig.2.8, used to record pixel-by-pixel images (microscopy and spectroscopy) and point spectra (spectroscopy) with high energy and spatial resolution. It consists of only the objective zone plate lens that focuses the x-rays to a ~30 nm spot on the sample. Scanning microscopes minimize the radiation dose to the specimen because the low (5-20%) efficiency zone plate is located upstream of the specimen rather than between specimen and detector. The sample is raster scanned pixel by pixel through the focal spot. In this case, the detector records the transmitted intensity for each pixel.

Scanning x-ray microscopes show their full power in combining the high spatial resolution imaging with high energy resolution for elemental and chemical state mapping.

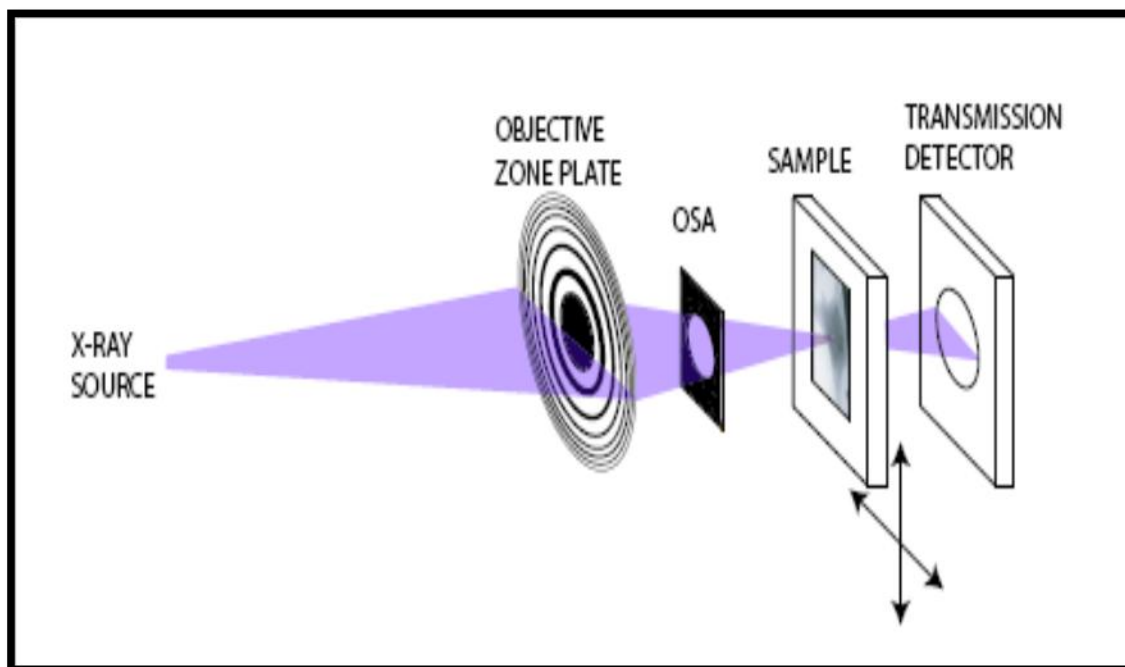


Figure 2.8. Schematic designs for scanning transmission x-ray microscopy. An objective zone plate is used to focus the x-rays on the sample. To generate an image, a thin section of the sample is raster-scanned in the focus of the x-rays under computer control. The transmitted photons are counted by an x-ray sensitive detector. OSA;order sorting aperture.

2.10.7.1 STXM and biological application

STXM has been used extensively to study biological samples (Kirz and Jacobson, 1995).

For instance, this technique has been used to quantitatively map the distribution of protein and DNA in bull sperm (Williams et al., 1995), and mapping the calcium distribution in tendon tissues (Figure 2.9; Buckley, 1995). Also, STXM has also been used to map the locations of

protein and DNA in sperm from different species of (Zhang et al., 1996; Buckley et al., 1997).

In microbial biofilms, mapping and speciation of metals (nickel, manganese, and ferrous iron) have been illustrated using STXM (Figures 2.10, 2.11 & 2.12; Dynes et al., 2006; Hunter, 2008).

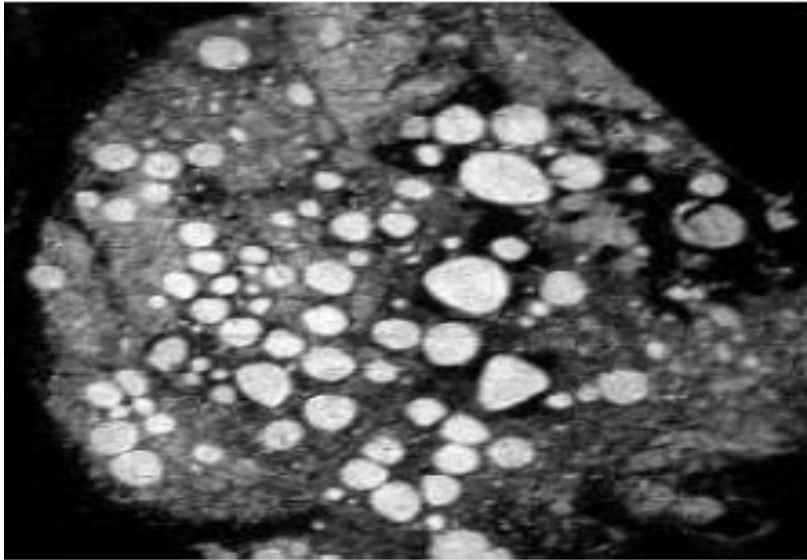


Figure 2.9. Map of calcium distribution in human tendon tissue.

White spots show the presence of calcium.

(Source: Buckley, 1995)

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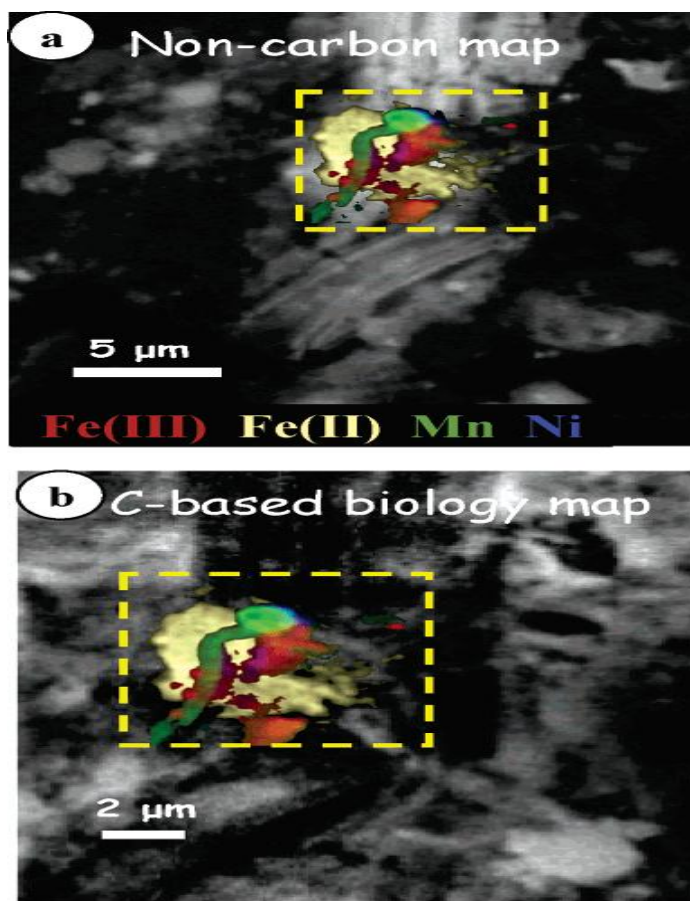


Figure 2.10. STXM micrograph of false color-coded composite map the metal species.

superimposed on (a) optical density (OD) image recorded at 282 eV (which highlights non-carbon components), and (b) OD difference image (288.2-282 eV) (Which highlights microbial entities) (pale yellow, Fe(II)-rich; red, Fe(III)-rich; green, Mn; blue, Ni; individual intensities scaled to fill each color range). The yellow dotted line indicates the area of the detailed metal study. (Source: Dynes, 2006)

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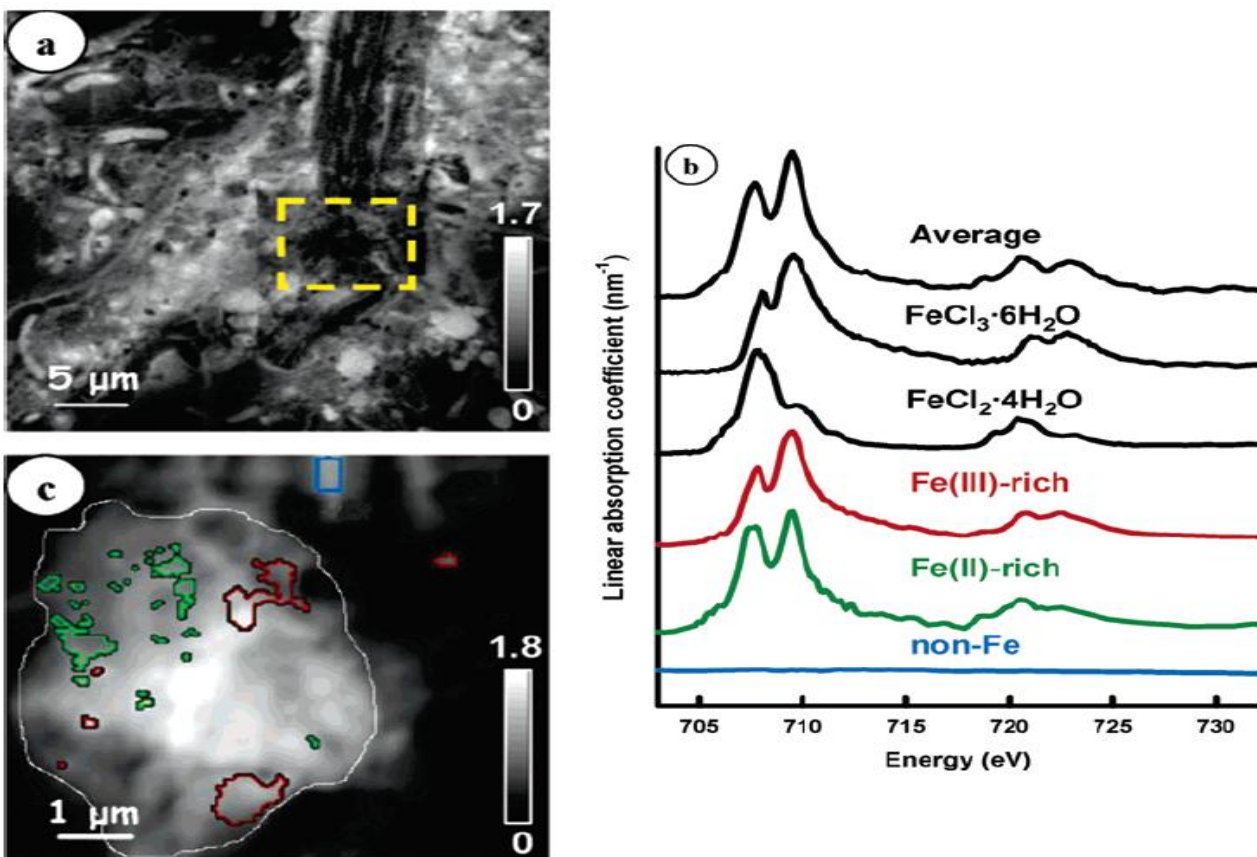


Figure 2.11. STXM micrograph and spectra for the speciation of metals in river biofilms.

- (a) The area of the detailed metals studies is indicated in the yellow box.
- (b) X-ray absorption spectra of the mapped metals in the area.
- (c) The yellow rectangle area is highlighted showing the speciation of metals.

(Source: Dynes, 2006)

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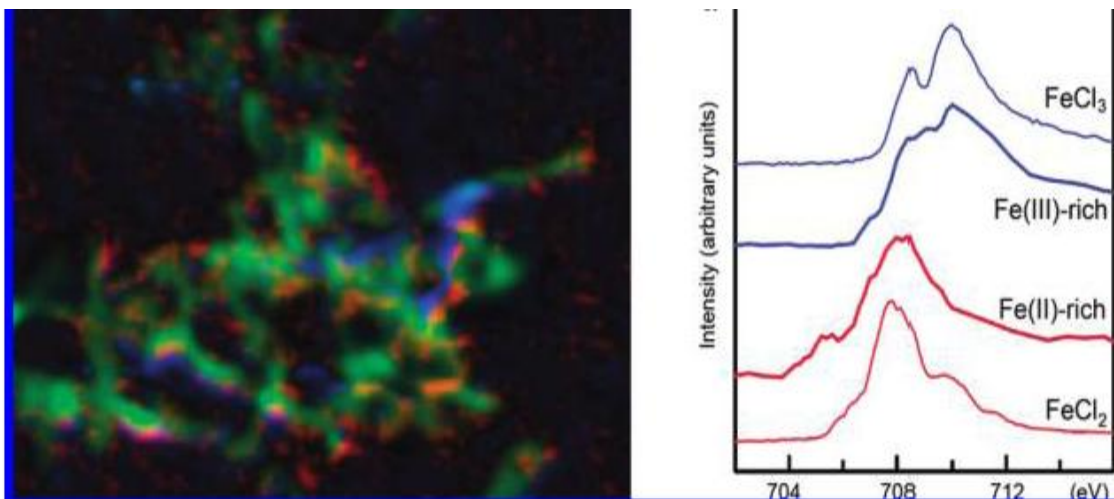


Figure 2.12. Mapping of iron speciation in bacterial biofilms.

(Source: Hunter, 2008)

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3.0 ALUMINUM LOADING CAUSES LIVER INJURY IN NEONATAL PIGLETS

3.1 Introduction

Aluminum is a ubiquitous element used extensively in modern daily life. Food, water, and pharmaceutical drugs are the major contributor for human exposure to aluminum (Neiboer et al., 1995; Yokel et al., 2001). In spite of its ubiquity, no biological function has so far been attributed to aluminum. For this reason, aluminum is considered a nonessential metal.

Aluminum was considered safe to humans until 1975, when high aluminum quantities in the brain tissues were found to be associated with encephalopathy in chronic dialysis patients (Alfrey and Solomons, 1976). This encouraged more research on the toxic effects of aluminum on humans. However, a wealth of research suggests that aluminum is toxic not only to humans but also to plants and animals (Ganrot, 1986; Yokel, 2000; Nayak, 2000; Pineros and Kochian, 2001).

Aluminum toxicity has been linked with neurological disorders such as Alzheimer's and Parkinson's diseases (Good et al., 1992; Yokel, 2000; Perl et al., 1982). In bones, aluminum overload is believed to be the predominant cause for the development of low-turnover bone disease Osteomalacia in dialysis patients (Ott et al., 1982; Nebeker and Coburn, 1986). Other health risks associated with aluminum toxicity includes microcytic anemia, cholestatic changes and alterations of the cytochromes P450 system (Klein et al, 1988; Bidlack et al., 1987).

The objective of this study is to elucidate and detect the early morphological and biochemical changes in the liver after the infusion of aluminum at high dose of 1500 µg/kg/day. This dose has been chosen because it has shown to cause cholestasis in neonatal piglets (Klein et al, 1987). In our lab, although this high dose has caused increase in serum total bilirubin and aluminum, no consistent histological changes were found in neonatal piglet's liver infused with

aluminum for three weeks (Li et al., 2005). However, the failure to detect the histological signs of cholestasis could be attributed to the low spatial resolution of light microscopy used in the study, which limits the identification of morphological ultrastructural changes in the liver. Light microscopy could not detect morphological structural changes in the liver such as loss of canalicular microvilli and swollen mitochondria (Shu et al., 1991; Cai et al., 2006). More sensitive imaging techniques are necessary to detect such ultrastructural changes in the liver. Hence, the study compared low-resolution light microscopy with the various imaging technologies currently available to determine which technique(s) may offer improved resolution and ability to detect early ultrastructural changes in the liver consistent with the development of cholestasis. The various imaging techniques employed to achieve this objective include light microscopy, transmission electron microscopy, Raman microscopy, confocal microscopy, and scanning transmission x-ray microscopy.

The results obtained will enable us to identify the suitable technique to study and understand the morphologic structural changes in the liver tissues that happen during the infusion of aluminum and compare these with changes seen in PN infused subjects. This information would help to develop a better understanding on the role of aluminum in causing liver injury.

3.2 Hypothesis and Objectives

The hypothesis of this study is that intravenous infusion of aluminum at a high dose (1500 µg/kg/d) to piglets causes liver injury with detectable early ultrastructural changes and increasing duration of aluminum exposure causes further deterioration in liver structure and function. Detecting early changes results in early interventions that may reduce the degree of liver injury.

The specific objectives for this study were to:

- 1-Evaluate the early morphological ultrastructural changes in piglet liver after the intravenous administration of aluminum at a dose of 1500 µg/kg/d.
- 2-Determine whether the morphological changes deteriorate further with increasing duration of exposure and whether these changes correlate with changes in biochemical markers of cholestasis.
- 3- Identify the appropriate imaging technique for studying the morphological ultrastructural changes in the liver associated with aluminum exposure.

3.3 Materials and Methods

3.3.1 Animals and Design

This study was conducted at the Health Science Building at the University of Saskatchewan. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Twenty neonatal piglets were obtained from the Prairie Swine Centre, Saskatoon. Piglets were taken from different sows at 2-4 days old and randomly assigned into:

- I- The Study Group consisted of 16 animals. In this group, piglets were randomly placed into four subgroups, each 4 piglets: one week, two weeks, three weeks, and four weeks group. Starting the day after surgery, every morning (9:00 am) the piglets were given daily intravenous injections of 1 mL/kg/day of aluminum chloride hexahydrate (1 mL containing 1500 µg aluminum). All study animals had free access to food and water.
- II- The Control Group consisted of 4 piglets. One piglet was assigned as control for each of the four study groups. These piglets were given daily injections of 1 mL/kg/day intravenous sodium chloride (0.9%) and had free access to food and water.

3.3.2 Surgery

Upon arrival, piglets were weighed and given milk replacer before the day of the surgery. On the day of surgery, the piglets of both study (n=16) and control (n=4) groups underwent surgery to insert a central venous access catheter. All procedures were carried out under general anesthesia in a certified operative facility at the Medical Research Building, University of Saskatchewan. Based on body weight (0.09 mg/kg), piglets were given approximately 0.03-0.06 mL intramuscular (i.m) Atropine (Glaxo Laboratories Inc., Toronto, Ontario, Canada) prior to induction of anesthesia. Anesthesia was induced with Ketamine (Rogarsetic, Rogar STB Inc., Montreal, Quebec, Canada), approximately 0.5 mL i.m based on weight (22 mg/kg) and Acetpromaxine (Atravet, Ayerst Laboratories, Montreal, Quebec, Canada) 0.5 mg/kg. Atropine and ketamine were combined in the same syringe. The piglets were maintained with spontaneous respiration and manually ventilated if apnea occurred. Anesthesia was maintained by isoflurane at rate of 1-1.5 %, oxygen and nitrous oxide at ratio of 2:1 with total flow of 1.0 mL/min. Approximately 0.1-0.2 mL of Excenel (Pharmacia Animal Health, Mississauga, Ontario, Canada), (50 mg/mL) at dose of 3 mg/kg/day was administered i.m as a preoperative prophylactic antibiotic. The piglets were placed in dorsal recumbancy and an incision of approximately 3 inches was made on the right side of the neck. A three French silicone venous access catheter (Fisher Scientific, Mississauga, Ontario, Canada) was inserted into the central venous system via the jugular vein and the catheter was tunneled under the skin to exit dorsally between the piglets shoulder blades. The neck incision was closed with 3-0 Novafil suture. Dorsal incision was a small puncture that was not closed with suture material. Piglets were given 6 mL sterile saline i.v through jugular vein. All the surgical procedures were carried out in a sterile environment using aseptic technique. The piglets were fitted with elastic bandages to protect the catheter. After daily administration of treatment solutions, the catheter was flushed

with one mL of saline and then one mL of heparinised 50% dextrose to maintain the patency of the catheter.

3.3.3 Care of animals

The piglets were housed in the Health Sciences Building, University of Saskatchewan in individual cages with a 12-hour light/dark cycle. The room temperature was maintained at 27°C with supplemental heat provided by heat lamps. All piglets were fed milk replacer only for two weeks. After two weeks, starter diet was added to the milk replacer for three and four week groups. All piglets were monitored daily for food intake, weight gain, and body temperature, color of mucus membranes, feces, and urine production. Each piglet had access to a toy for entertainment. The patency of catheter was maintained by daily flushing with 0.9% sodium chloride solution (Abbott Laboratories, Montreal, Quebec, Canada). Piglets were weighed each morning and injections of either aluminum chloride hexahydrate for the study group or sodium chloride solution (0.9%) for the control group were prepared accordingly. The incisions were cleaned daily until healed and the sutures were removed on postoperative day 7. The piglets were fitted with elastic bandages to protect the catheter. Cages were cleaned daily.

3.3.4 Preparation of aluminum injections

The aluminum injection was in the form of aluminum chloride hexahydrate (Fisher Scientific, Mississauga, Ontario, Canada). To prepare the aluminum injections with a final concentration of 1500 µg/mL, 3.3542 g of aluminum chloride hexahydrate was dissolved in 10 mL of sterile water. Then, the solution was transferred into an empty 250 mL intravenous bag (Abbott Laboratories, Canada) and mixed with normal saline to 250 mL. Starting the day after the surgery, piglets were weighed each morning at 9:00 am and injections of either aluminum chloride hexahydrate for study group or sodium chloride solution (0.9%) for control group were

prepared accordingly. The injections for both groups were given once daily for the whole period of the study.

3.3.5 Sample collection and analysis

Daily weight was recorded. Blood samples (5 mL) were drawn by syringe from the vascular access catheter into a metal-free vacutainer tubes from piglets at baseline (prior to aluminum and sodium chloride administration) and at the end of the aluminum exposure period for each subgroup (7, 14, 21, or 28 days). The blood was centrifuged, and the obtained serum was aliquotted into three parts. The aliquots were each used to measure the following: 1) direct bilirubin using a QuantiChrom bilirubin assay kit (Bioassay Systems, Hayward, CA, USA), and spectrophotometric analysis according to manufacturer instructions, 2) total bile acids in the serum using a BioQuant bile acid assay kit (San Diego, CA, USA) and spectrophotometric analysis according to manufacturer instructions, and 3) aluminum concentration using a inductively coupled plasma mass spectrometry (Thermo X Series ICP-MS, Thermo Fisher Scientific Inc, Waltham, MA, USA) at the Saskatchewan Research Council (SRC), Saskatoon, SK.

The ICP-MS sample preparation and analysis was done according to the method described in Standard Methods for the Examinations of Water and Wastewater, 2005. At the end of the aluminum exposure period for each subgroup, piglets were anesthetized with 5% isoflurane in 100% oxygen, and blood sample was collected via cardiac puncture, and then sodium pentobarbital was injected via cardiac puncture into the heart. Once the piglet was deceased, urine from the bladder and bile from the gallbladder were collected using syringe needles. Samples from urine, bile, blood, and liver were quickly collected in metal free plastic containers and sent to SRC for aluminum determinations by ICP-MS. For histopathological

observation, liver sections were cut and processed for light microscopy, transmission electron microscopy (TEM), scanning transmission x ray microscopy (STXM) Raman microscopy, and confocal microscopy as indicated below. The remaining liver tissues were snap frozen and stored at -80°C for further studies.

3.3.6 Inductively coupled plasma-mass spectrometry (ICP-MS)

Inductively coupled plasma-mass spectrometry can measure the presence of more than 75 elements in a single scan, and can achieve detection limits as low as parts per trillion (ppt) levels for many elements (Keeler, 1991). Aluminum level in blood serum was detected down to 0.001 µg/mL, and in urine to 0.02 µg/mL using ICP-MS (Ward, 1989). In liver, kidney, and brain, the quantification of aluminum was detected at levels as low as 0.04 µg/g (Owen et al. 1994). Using ICP-MS at the Saskatchewan Research Center (SRC), Saskatoon, the quantification limit for aluminum in liver is 0.05 µg/g. In urine, bile and serum, the quantification limit for aluminum is 0.05 µg/L.

3.3.7 Microscopic and imaging techniques sample preparation

In order to achieve the objective of identifying an imaging technique to study the structural changes associated with aluminum loading as well as detection of aluminum deposition, the following imaging techniques were utilized: Raman microscopy, confocal microscopy, transmission electron microscopy, and scanning transmission electron microscopy. However, light microscopy was used in all experiments as the conventional technique to evaluate the histologic changes in the liver.

3.3.7.1 Light microscopy sample preparation

Liver samples were fixed and stored in 10% buffered formalin at room temperature. The fixed sections were trimmed and transferred to coded plastic histology cassettes (Tissue-Tek, Miles. Etobicoke, ON, Canada). Tissues were dehydrated in a graded series of ethanol baths (1

hour in each reagent): 2x 70% ethanol, 2x 95% ethanol, 2x 100% ethanol, and 2x 100% xylene, using a tissue processor. Tissues then were embedded in the paraffin wax. The blocks were trimmed, chilled on ice, and sectioned at 5- μ m thickness on a rotary microtome. Sections were dried in the oven at 37°C for 24 hours. The liver specimens were stained for routine light microscopy with hematoxylin and eosin (H&E) staining, cover slipped and observed using a light microscope for structural changes.

3.3.7.2 Confocal microscopy sample preparation

Liver samples were prepared and processed (see sample preparation below) in the Department of Anatomy and Cell Biology, University of Saskatchewan. Analysis of the liver tissues to evaluate the intrahepatic distribution of aluminum samples was carried out at Saskatchewan Structural Sciences Centre (SSSC), University of Saskatchewan.

Liver samples were fixed with 70% ethanol for 2 days at room temperature and were immersed in pure ethanol for 2 days, followed by embedding in methyl methacrylate resin. Thin sections (10- μ m thickness) were produced manually using a microtome. Liver sections were stained in acetate buffer (pH 4.0) containing 2.5×10^{-5} mol/L lumogallion for 1 hour at 70°C. The specimens were then examined for aluminum deposition using a confocal microscope.

3.3.7.3 Raman microscopy sample preparation

The liver samples were analyzed using Raman microscope at the Saskatchewan Structural Science Center (SSSC), University of Saskatchewan.

For Raman microscopy sample preparation, frozen liver sections were warmed to room temperature and rinsed with phosphate-buffered isotonic saline solution (pH 7.4). Liver specimens (thickness about 1 mm) were cut and placed in a chambered coverslip, and immersed in normal saline solution and examined using the Raman microscope.

3.3.7.4 Transmission electron microscope - energy dispersive microanalysis (TEM-EDX) sample preparation

The fixation and embedding of the liver samples was done in the College of Pharmacy and Nutrition, University of Saskatchewan. The liver samples were processed as follow: liver sections were cut with no larger than 1mm³ with a sharp razor blade. The sections were fixed primarily with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and kept in the refrigerator for 4 hours. The liver sections were washed with the sodium phosphate buffer three times for 30 minutes each. Then, the samples were fixed (secondary fixation) with 4% osmium tetroxide in 0.1 M sodium phosphate buffer and kept in the refrigerator for 4 hours. The samples were washed again with cold sodium phosphate buffer for 15 minutes and were dehydrated in a graded series of ethanol (25%, 50% 70-75%, 90-95%, 100%) for 10 minutes each. Then, the samples were washed in distilled water for 30 minutes. The samples were infiltrated with the following ratios of resin:solvent (1:2, 1:1, 2:1, 1:0) for one hour each. The samples were embedded in 100% resin in small plastic capped vials. The plastic vials were placed in oven at 70 °C for more than 8 hours until the resin is completely polymerized. Samples were shipped to the facility of Electron Microscopy, Faculty of Health Sciences, McMaster University to be cut and observed for morphological changes and locating aluminum deposition in the liver samples. Transmission electron microscope was equipped with EDX to locate and determine the nature of the electron dense materials observed in liver tissues. We chose to ship the samples to facility of Electron Microscopy, Faculty of Health Sciences, McMaster University because their TEM was equipped with EDX. Here, at the University of Saskatchewan, at that time, no TEM equipped with EDX.

3.3.7.5 Scanning Transmission X-ray Microscopy (STXM) Sample Preparation

Since there is no standard technique for STXM liver sample preparation, we followed a similar standard technique used for electron microscopy. Two sample preparation techniques

were followed; 1) the same as TEM sample preparation but unfixed (no gluteraldehyde and osmium tetroxide), and the 2) samples were immersed in 70% alcohol only and kept at room temperature until shipped to the facility of Electron Microscopy, Faculty of Health Sciences, McMaster University. For preparing the unfixed samples, small pieces of liver tissues (1x1x1 mm) were cut randomly from different liver areas. The tissues were dehydrated with different concentration (25%, 50% 70-75%, 90-95%, 100%) of ethanol for 20-30 minutes each. Then, the tissues were embedded in epoxy resin, polymerized at 60°C for 24 hours, and sent later to the facility of Electron Microscopy, Faculty of Health Sciences, McMaster University. At McMaster University, the liver tissues were cut using ultramicrotome into 90 nm thick. Sections were collected and mounted on copper grids and shipped to the Canadian Light Source (CLS) (Saskatoon, Canada) for STXM analysis.

3.3.8 Statistical analyses

Descriptive statistics were performed for the variables. Results were expressed as mean \pm standard deviation (SD). One-way ANOVA and post hoc test (Student-Newman-Keuls) were used to compare the variables in different groups. However, when the variance was not homogenous, values were expressed as median and interquartile range (IQR) and a non parametric test (Mann-Whitney U test) was used to compare the difference between the groups. The correlation between the variables was evaluated using Pearson correlation. A p-value less than 0.05 were considered significant.

3.4 Results

Piglets of the control group (n=4) were assigned randomly into four different period groups as follow: one, two, three, and four week. One piglet was assigned as control for each study group. However, during the experiment, only piglets for one week and two weeks finished

the period of the study. For three and four week piglets, because of catheter-related problems, we had to sacrifice the control piglets after one week of the infusion.

3.4.1 Body weight

Aluminum infusion was associated with slight decrease in body growth rate of piglets throughout the duration of the study (Fig.3.1). The highest body growth rate was noticed in the study one week group (8%) and lowest growth rate was seen in the study four weeks group (5.3%). No significant difference was found between the groups.

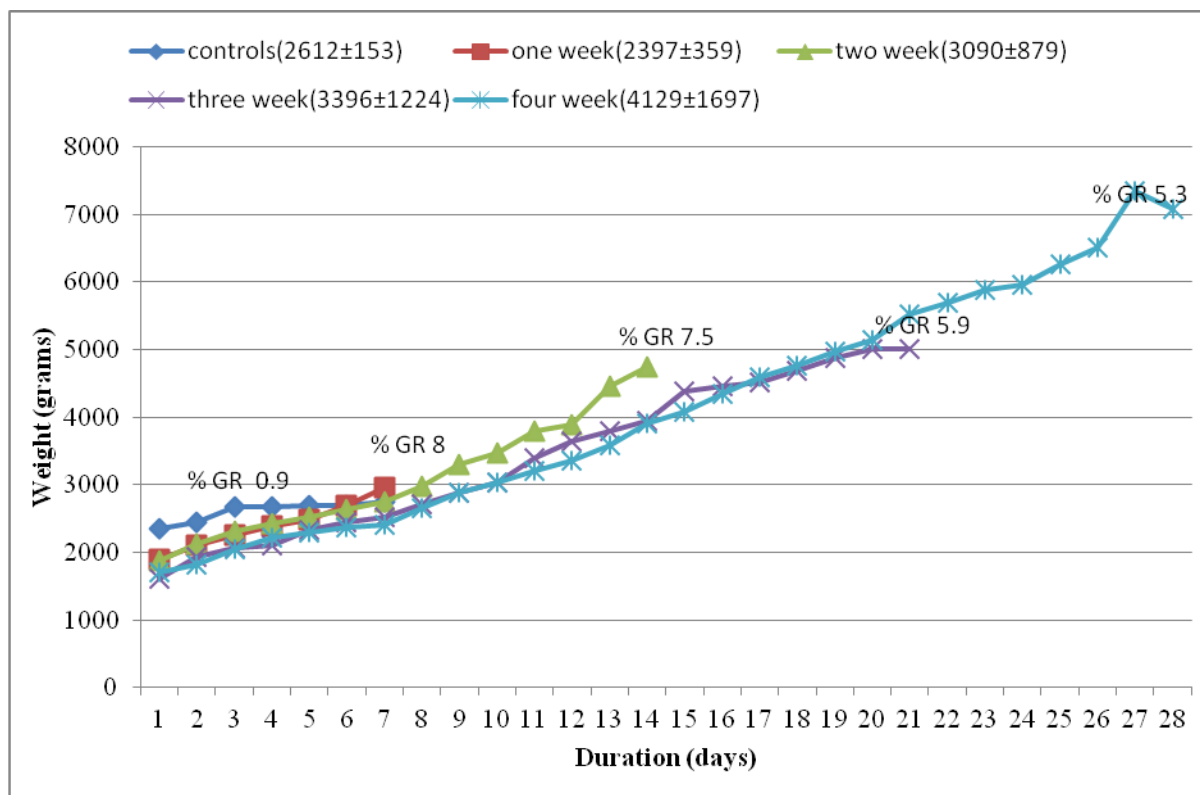


Figure 3.1. Weight patterns of piglets during the experiment

Data are expressed as mean \pm SD between brackets at the top of the graph. Data series labelled with the % growth rate. Number of piglets in each group (n=4). % GR: % growth rate.

3.4.2 Biochemical assays

3.4.2.1 Serum direct bilirubin

The administration of aluminum in the piglets was associated with slight increase in the serum direct bilirubin level (Table 3.1). Statistically, there is no difference in serum direct bilirubin level among controls at day 0 and day 7. However, the increase in serum direct bilirubin level was significant at day 7 in the study group compared to day 0 and day 7 of controls, and day 0 of study group (Table 3.1). The level of the serum direct bilirubin peaked at day 21 of infusion and declined at the day 28 of the infusion. However, the decline in the serum direct bilirubin level at day 28 was not statistically significant compared to the other groups.

Table 3.1. Results of direct bilirubin ($\mu\text{mol/L}$) in all groups.

Group	Median (IQR)	Mean \pm SD
Control group		
Day 0 (n=4)	2.0 (1.0-7.0) ^a	3.4 \pm 3.2
Day 7 (n=4)	2.6 (1.5-7.6) ^a	3.9 \pm 3.5
Study group		
Day 0 (n=16)	4.1 (2.8-6.8) ^a	5.0 \pm 3.2
Day 7 (n=16)	7.0 (3.6-11.5) ^b	7.9 \pm 4.2
Day 14 (n=11) ¹	8.2 (3.4-17.2) ^{b,c}	9.6 \pm 6.6
Day 21 (n=8)	9.9 (2.5-15.3) ^{b,c}	9.1 \pm 6.4
Day 28 (n=4)	5.1 (4.8-7.0) ^{a,b}	5.6 \pm 1.2

Values are expressed as median and interquartile range (IQR). Medians with different superscripts (a, b,c) were significantly different ($p<0.05$) when compared to each other using a non parametric test (Mann-Whitney U test).

1-Insufficient sample (piglet # 51, day 14).

3.4.2.2 Serum total bile acids

The median level of serum total bile acids was increased in all the study groups throughout the duration of aluminum infusion (Table 3.2). The aluminum loaded piglets had significantly ($p<0.05$) increased levels of serum total bile acids at day 14, 21, and 28 of the study compared to controls at day 0 and 7, and study group at day 0. The highest increase in the serum total bile acids level of study group was seen at day 28 of aluminum infusion (Table 3.2).

Table 3.2. Results of serum total bile acids ($\mu\text{mol/L}$) in all groups.

Group	Median (IQR)	Mean \pm SD
Control group		
Day 0 (n=4)	6.5 (5.0-11.5) ^a	7.6 \pm 4.0
Day 7(n=4)	11.8 (7.0-13.9) ^a	10.9 \pm 4.0
Study group		
Day 0 (n=16)	6.7 (4.6-11.4) ^a	13.1 \pm 18.0
Day 7 (n=16)	19.0 (12.1-25.4) ^{a,b}	19.9 \pm 16.0
Day 14 (n=12)	29.3 (19.2-50.4) ^{b,c}	33.1 \pm 16.0
Day 21 (n=8)	36.4 (19.4-56.2) ^{b,c}	37.7 \pm 19.0
Day 28 (n=4)	48.9 (43.9-56.9) ^{c,d}	49.9 \pm 7.0

Values are expressed as median and interquartile range (IQR). Medians with different superscripts (a,b,c,d) were significantly different ($p<0.05$) when compared to each other using a non parametric test (Mann-Whitney U test).

3.4.2.3 Aluminum content of serum, liver, bile, and urine

The median level of serum aluminum content was significantly increased at day 7, 14, 21, and 28 ($p < 0.05$) in study group compared to day 0 and 7 in the controls and day 0 of the study

group (Table 3.3). For the effect of aluminum loading on the liver, the hepatic aluminum content (Table 3.4) was proportionally increased with the duration of the study. The increase in the hepatic aluminum content was significant ($p < 0.05$) among all the study groups. No correlation was found between serum aluminum content, direct bilirubin, and serum total bile acids. However, the hepatic aluminum content was positively correlated (Figure 3.2) with the serum total bile acids ($r = 0.67$, $p = 0.001$).

Table 3.3. Serum aluminum concentration ($\mu\text{g/L}$) in all groups.

Group	Median (IQR)	Mean \pm SD
Control group		
Day 0 (n=4)	25.0 (25.0-25.7) ^a	25.0 \pm 0.5
Day 7 (n=4)	108 (62.0-615) ^b	261.6 \pm 346
Study group		
Day 0 (n=16)	84.0 (25.0-290) ^c	162 \pm 169
Day 7 (n=16)	1013 (754-1500) ^d	1125 \pm 124
Day 14 (n=12)	1150 (597-1550) ^d	1175 \pm 647
Day 21 (n=8)	990 (574-1436) ^d	1313 \pm 1058
Day 28 (n=4)	1272 (340-1862) ^d	1159 \pm 797

Values are expressed as median and interquartile range (IQR). Medians with different superscripts (a,b,c,d) were significantly different ($p < 0.05$) when compared to each other using a non parametric test (Mann-Whitney U test).

Table 3.4. Hepatic aluminum concentration (µg/g) in all groups.

Group	Median (IQR)	Mean±SD
Control group (n=4)	0.05 (0.05-0.14) ^a	0.08±0.06
Study group		
One week (n=4)	25.5 (15-28) ^b	22.7±7.5
Two week (n=4)	44.0 (30-69) ^c	47.7±21.0
Three week (n=4)	53.5 (43-78) ^c	58.5±19.0
Four week (n=4)	81.5 (78-85) ^d	81.7±3.8

Values are expressed as median and interquartile range (IQR). Medians with different superscripts (a,b,c,d) were significantly different ($p<0.05$) when compared to each other using a non parametric test (Mann-Whitney U test).

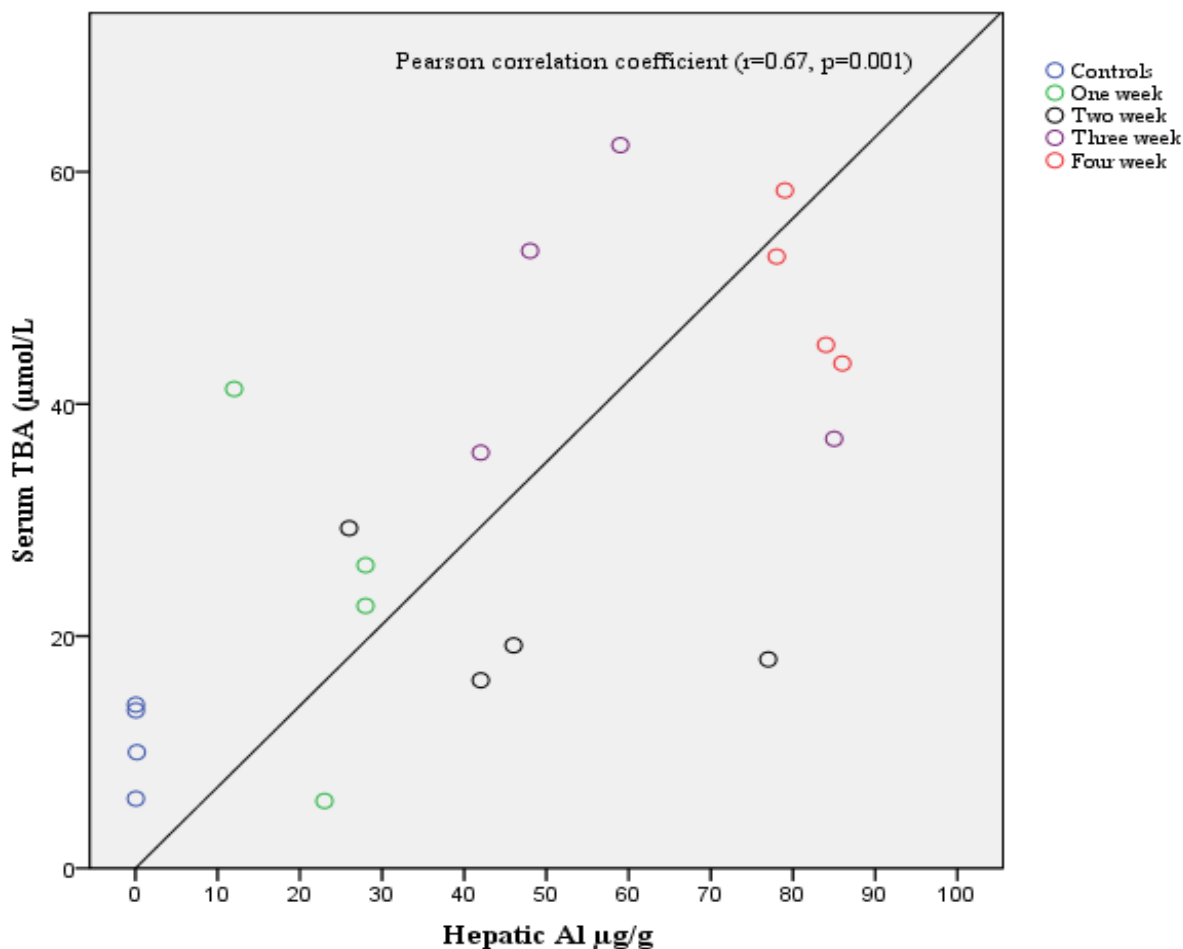


Figure 3.2. Correlation between hepatic aluminum and total serum bile acids (TBA).

The scatter plot represents the correlation between the concentration of hepatic aluminum and the serum total bile acids in the control piglets ($n=4$) and the piglets of four study groups (each has 4 piglets). The controls were given daily injection of 1 mL/kg/day of sodium chloride (0.9%). The study groups were given daily injections 1 mL/kg/day of aluminum chloride hexahydrate (1 mL contains 1500 µg aluminum).

In urine, the median level of aluminum content was proportionally increased in all study groups throughout the experiment (Table 3.5). The increase in the urine aluminum content was significant ($p<0.05$) in the third and fourth week groups compared to the controls and one week study group. In bile, the median levels of aluminum concentrations were increased in the one

and two week group of experiment and declined in the third week group. However, the aluminum content in bile was elevated again at the fourth week group (Table 3.6).

Table 3.5. The concentration of aluminum (µg/L) in urine in all groups.

Group	Median (IQR)	Mean ± SD
Control (n=3) ¹	61.2 (51-155) ^a	120±112
Study group		
One week (n=4)	1020.0 (635-1377) ^a	995±733
Two week (n=4)	1320.0 (560-4450) ^{a,b}	2487±3117
Three week (n=4)	4335.0 (2652-6324) ^b	4444±2126
Four week (n=4)	4913.0 (1105-8262) ^b	4643±4140

Values are expressed as median and interquartile range (IQR). Medians with different superscripts (a, b) were significantly different ($p < 0.05$) when compared to each other using a non parametric test (Mann-Whitney U test).

1. Urine sample was not obtained from piglet # 54 (control), the bladder was empty.

Table 3.6. The concentration of aluminum (µg/L) in bile in all groups.

	Median (IQR)	Mean ± SD
Group		
Controls (n=4)	51 (31-51) ^a	44.5±13.0
Study group		
One week (n=4)	140 (51-372) ^{a,b}	188±176
Two week (n=4)	227 (198-305) ^b	243±60
Three week (n=4)	66 (51-196) ^{a,b,c}	104±87
Four week (n=4)	146 (97-157) ^{c,d}	133±35

Values are expressed as median and interquartile range (IQR). Medians with different superscripts (a, b,c,d) were significantly different ($p < 0.05$) when compared to each other using a non parametric test (Mann-Whitney U test).

3.4.3 Histopathological observation

In this study, I aimed to identify an appropriate imaging technique(s) for the early detection of ultrastructural changes consistent with cholestatic changes of the liver. Several imaging techniques were employed throughout the study, namely light microscopy, transmission electron microscopy equipped with energy dispersive microanalysis (TEM-EDX), Raman microscopy, confocal microscopy and scanning transmission x-ray microscopy (STXM).

3.4.3.1 Light microscopy

Light microscopy was used as the starting imaging tool to identify any histopathological features seen after the infusion of Al in the piglets. Liver samples from the study and control groups were evaluated for histologic changes blindly by a board- certified Pathologist at the Prairie Diagnostics Center in the Western College of Veterinary Medicine. Inflammation, necrotic foci, and hydropic degeneration were the only changes seen in the livers of the piglets (Table

3.7). Morphological evidence characterized with inflammation was seen in all groups.

Inflammation in the liver samples was more evident after four weeks of AI infusion. No bile plugs were seen in any study or control piglet livers.

Table 0.7. Histological observations of piglet liver using light microscopy.

Group	Inflammation	Necrotic foci	Bile duct proliferation	Portal fibrosis	Hydropic degeneration	Bile plugs
Control (n=4)	1	0	0	0	0	0
One week (n=4)	1	0	0	0	1	0
Two week (n=4)	2	0	0	0	0	0
Three week (n=4)	2	0	0	0	0	0
Four week (n=4)	3	1	0	0	0	0

Numbers (0-3) represent number of subjects.

3.4.3.2 Raman and Confocal microscopy

Frozen piglet liver samples from previous work were cut, prepared, and examined for AI deposition and morphological changes in the microscope facility at Saskatchewan Structural Science Center. The results showed no clear histological features and no AI deposition was seen in the liver samples. Although significant work was done using Raman and confocal microscopy in biological sciences, in our project, we could not utilize the techniques to achieve our objectives. The unsuccessful use of Raman and confocal microscopy to detect AI deposit in the liver tissues attributed to the lack of expertise as the scientists who run the confocal and Raman microscopes were not familiar with biological samples. Another reason is that the liver samples

examined were stored improperly, which caused unclear morphological structure to the liver sections. It is worthwhile to mention that Raman microscopy has been used successfully to distinguish between normal and cancer cells, and to differentiate between different types of tumor cells (Crow et al., 2005; Choi et al., 2005; Jong et al., 2006), and utilizing this imaging technique properly with the aid of expertise personnel in the future could help researchers, especially in the area of PNAC to distinguish between normal and injured liver, and could detect any early structural especially in the livers of patients receiving PN.

Using both techniques, neither clear morphology nor aluminum deposition was seen in the piglet livers using these techniques. The other techniques to accomplish my objective were explored.

3.4.3.3 Transmission electron microscopy energy dispersive spectroscopy (TEM-EDX)

Transmission electron microscopy equipped with energy dispersive spectroscopy demonstrated the presence of aluminum deposits in the lysosomes of hepatocytes of the study group (Figure 3.3). Morphological changes, as evidenced by the loss of canalicular microvilli (Figure 3.4) and condensation of mitochondria, were seen in all but the control group. The loss of the canalicular microvilli was observed in the two, three, and four week of infusion groups but not in the one week group and controls. In Table 3.8, the results of semi-quantitative analysis of the liver tissues for elemental distribution using EDX showed that aluminum deposition was proportionally increased with the duration of infusion. The highest aluminum levels were observed in the four-week group and the lowest was in the control group (Table 3.8).

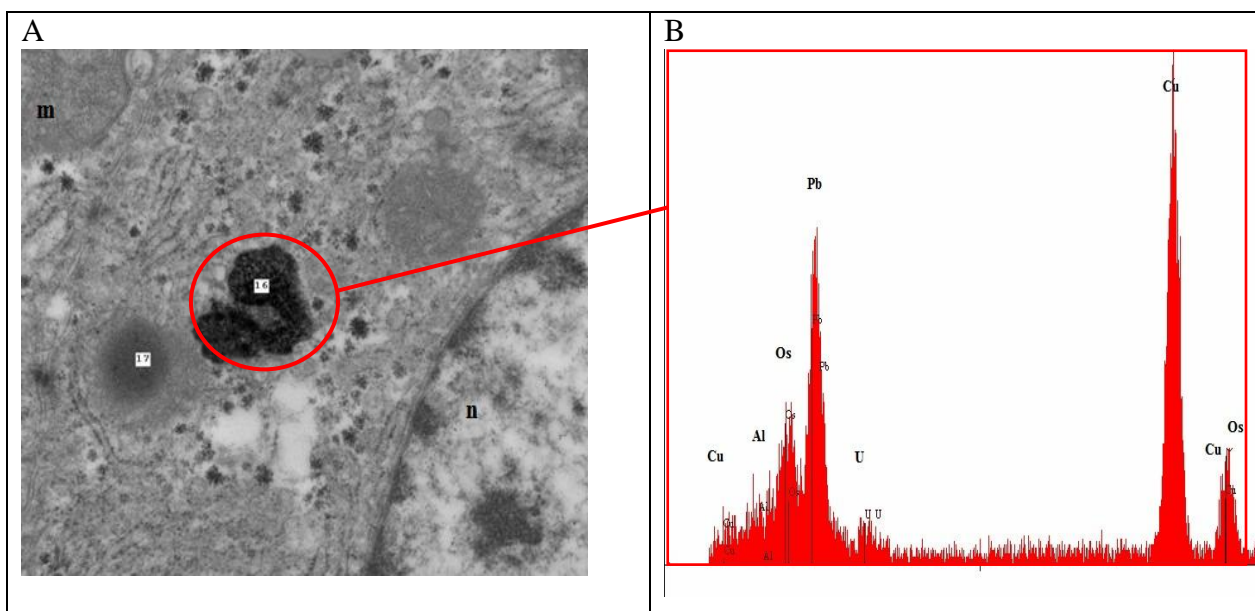


Figure 133. Electron micrograph and EDX spectra for piglet liver from the study group.

a) Electron micrograph of liver of piglet from the study group receiving daily injection of 1 mL/kg/day of aluminum chloride hexahydrate (1 mL 1500 μ g Al), x50000. b) EDX spectra taken from the electron dense in the hepatocyte showing the elemental distribution. Peaks of aluminum (Al), copper (cu), osmium (Os), uranium (U), and lead (Pb) were observed. The peaks of Cu are originated from the grids. Os, Pb, U peaks are from fixative and staining materials. m: mitochondria, n: nucleus.

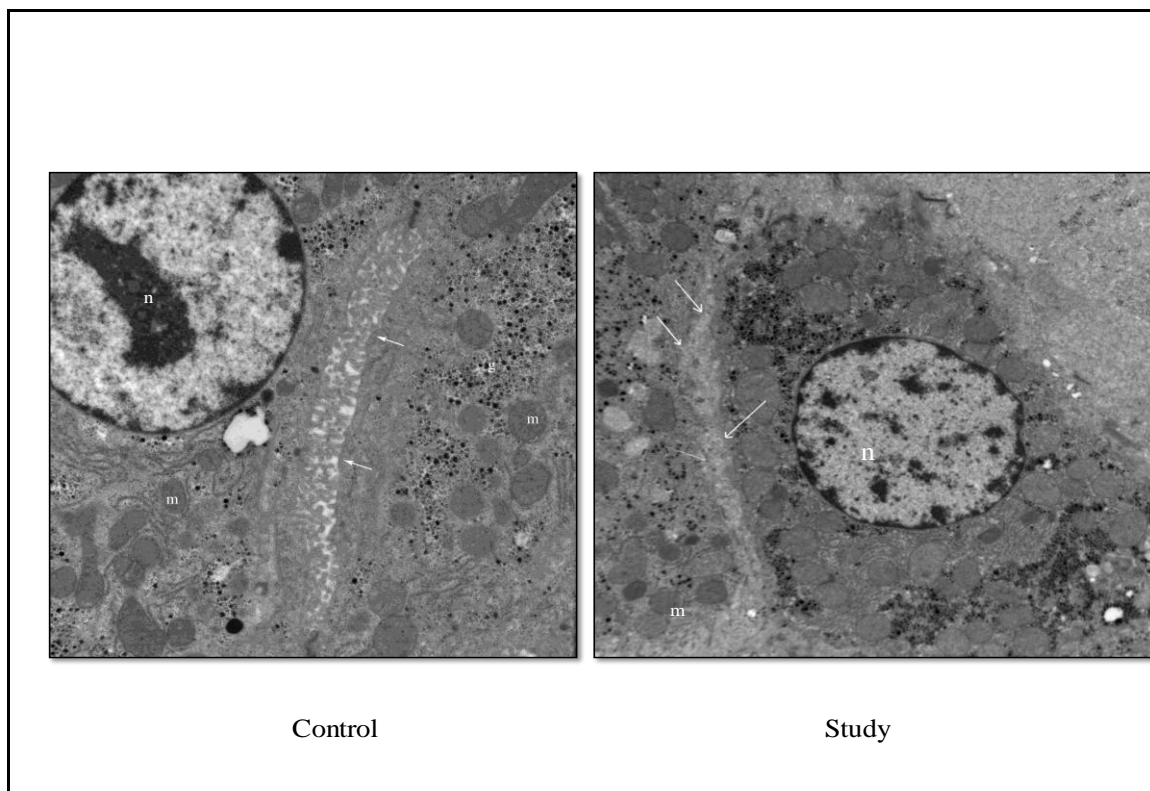


Figure 3.4. Electron micrographs for the liver of control and study piglets.

The electron micrograph of the control piglet show intact microvilli in the bile canaliculi (arrow), x 15000. The control piglets were given daily injections of mL/kg/day of sodium hydroxide (0.9%). A marked loss of microvilli of bile canaliculi was seen in the study group of piglets given daily injections of 1 mL /kg/day of aluminum chloride hexahydrate (each 1 mL contains 1500 μ g Al), x10000. m: mitochondria, n: nucleus.

Table 0.8. Results of microanalysis using energy dispersive x-ray microanalysis (EDX) of piglet's liver presented as peak/background ratios.

Element	Al	Cu	Os	P	Si	Fe	S
Group							
Controls (n=2)	0.3±0.1	11.5±3.7	7.4±1.1	2.1±0.4	0.5±0.1	2.6±1.2	0.49±0.25
One week (n=2)	0.3±0.1	12.0±11.0	2.5±2.9	0.8±0.9	0.6±0.3	0	0
Two week (n=1)	0.4±0.2	14.8±3.2	7.8±1.3	2.1±0.8	0.1±0.3	0	0.11±0.24
Three week (n=2)	0.5±0.2	8.8±2.1	5.9±1.4	2.6±0.8	0.3±0.3	4.10±3.51	0.35±0.26
Four week (n=1)	0.6±0.1	19.1±2.1	0	1.7±0.5	0.5±0.1	1.35±0.95	0

Al: aluminum, Cu: copper, Os: osmium, P: phosphorus, Si: silicone, S: sulphur. Means are the average of the values collected from 6 different sites in the liver tissues for each animal.

3.4.3.4 Scanning transmission x ray microscopy (STXM)

The scanning transmission x-ray microscopy (STXM) was also used in our project as one of the prospective advanced imaging techniques to achieve my objectives. The aim was to identify any early morphological changes and to speciate Al in the liver samples. The piglet liver samples were cut, fixed and prepared using the same sample preparation technique as Transmission electron microscopy and examined by the beamline scientist at STXM. The results showed no clear morphological structures were seen (Figure 3.5). Also, Al could not be detected in the liver samples. The failure to detect Al and the unclear morphology led us to try different sample preparation techniques. At this time, unfixed liver samples were cut, prepared and examined using STXM. The results showed damaged tissues and no Al was seen (Figure 3.6). The damage was attributed to the lack of fixative in the tissue.

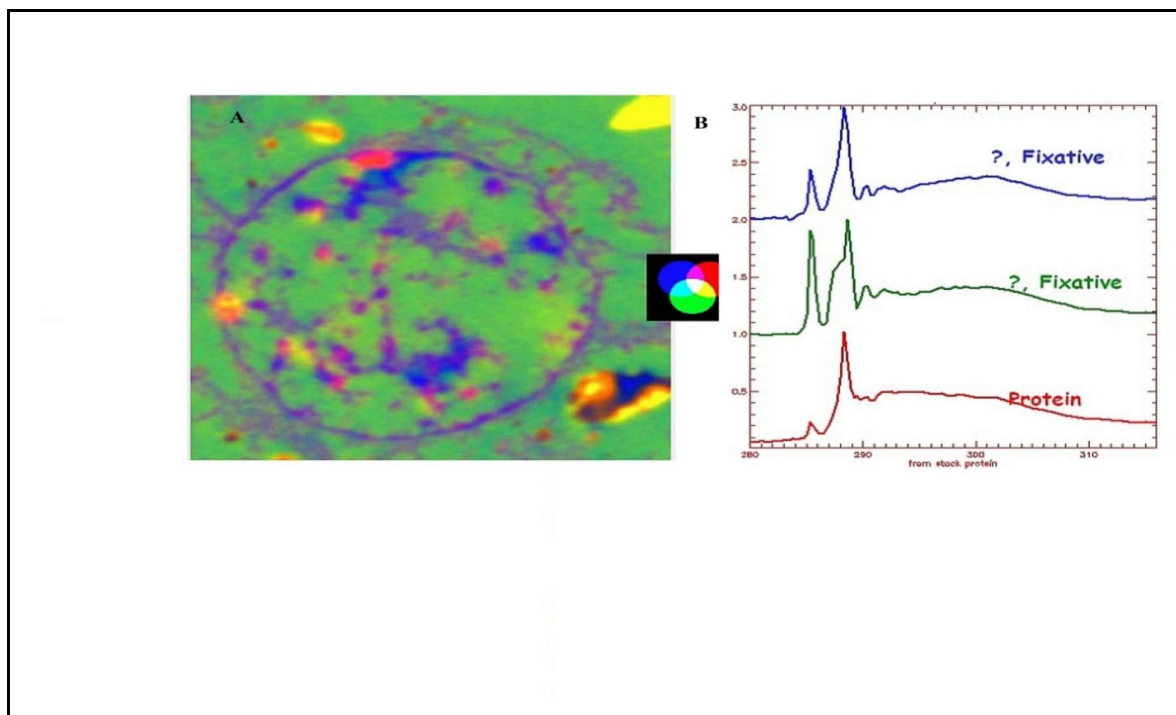


Figure 3.5. (A) Scanning transmission x-ray microscopy color-coded composite piglet liver.

(B) Spectra extracted from the image demonstrating the distribution of materials in the liver and showing no aluminum was found in the liver of the piglet from the study group. Piglets of the study group were given daily injections of 1 mL/kg/day aluminum chloride hexahydrate (1 mL contains 1500 μg Al). A blue and green color spectrum represents two different fixatives, and red color spectra represent the protein distribution. Two fixatives were used in liver tissues; glutaraldehyde and osmium tetroxide.

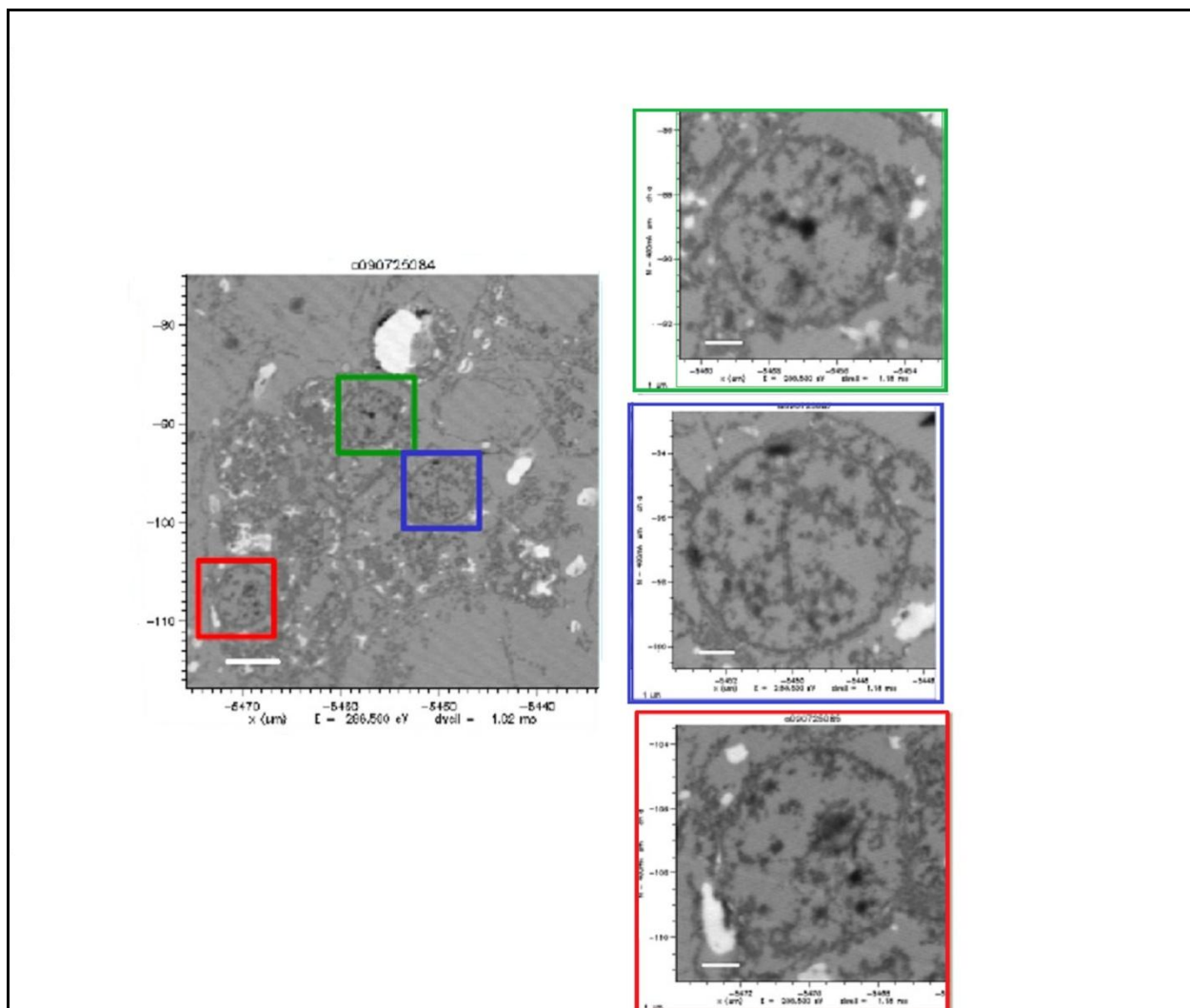


Figure 3.6. Scanning transmission x-ray microscopy images of unfixed liver tissues taken from piglet of the study group.

Piglets of the study group were given daily injections of 1 mL/kg/day aluminum chloride hexahydrate (1 mL contains 1500 μg Al). The unfixed liver sections were damaged and no clear morphological structure was seen.

3.5 Discussion

A neonatal piglet model was successfully developed to investigate the effect of aluminum loading on the biochemical parameters and histopathological features of the liver. The main objective of this study was to elucidate the early biochemical and ultrastructural changes in the liver that are associated with the parenteral administration of high aluminum dose (1500 µg/kg/d). This dose has been chosen because it has shown to cause cholestasis in neonatal piglets (Klein et al, 1987). In our lab, although this high dose has caused increase in serum total bilirubin and aluminum, no consistent histological changes were found in neonatal piglet livers infused with aluminum for three weeks (Li M, 2005). However, the failure to detect the histological signs of cholestasis could be attributed to the low spatial resolution of light microscopy used in that study which limits the identification of morphological ultrastructural changes in the liver. Light microscopy could not detect morphological structural changes in the liver such as loss of canalicular microvilli and swollen mitochondria (Shu et al., 1991; Cai et al., 2006). More sensitive imaging techniques are necessary to detect such ultrastructural changes in the liver. Hence, the study compared low resolution light microscopy with the various imaging technologies currently available to determine which technique(s) might offer improved resolution and ability to detect early ultrastructural changes in the liver consistent with the development of cholestasis. The various imaging techniques employed to achieve this objective include light microscopy, transmission electron microscopy, Raman microscopy, confocal microscopy, and scanning transmission x-ray microscopy.

In our study, using Raman and confocal microscopy, neither clear morphology nor aluminum deposition was seen in the piglet livers using these techniques. The unsuccessful use of both techniques in our project attributed mainly to the storage condition of liver samples used. The liver sections (from previous work) were frozen at -20°C. The two commonly used methods

of tissue long-term storage are either paraffin embedding or snap-freezing method. In both methods, excised tissues need to be processed immediately to avoid ultrastructural morphological damage to the tissue. Unfortunately, at that time, we did not have fresh liver tissues and we had to use the stored liver tissues. Another reason is that the scientists at Saskatchewan Structural Science Center are not familiar with biological samples.

Scanning transmission x-ray microscopy (STXM) has the potential to reveal ultrastructure of intact and hydrated cells at high spatial resolution (~ 30 nm) (Jacobson, 1999). The spectroscopy associated with microscopy can be used to identify and reveal the different states of different elements present in the sample. Scanning transmission x-ray microscopes show their full power in combining the high spatial resolution imaging with high-energy resolution for elemental and chemical state mapping. Unfortunately, in our work, we could not benefit from the powerful imaging characteristics of STXM. The unsuccessful attempts of benefiting from STXM technique in our project could be attributed into two main reasons. The first reason is because of the competitiveness at the Synchrotron and the limited number of applications accepted in the beam line which limited our chance of using this technique. The second reason is the lack of proper sample preparation especially liver samples loaded with aluminum. No STXM standard sample preparation for liver tissues is yet established. In this work, liver sample were processed in two different ways. One using the same sample preparation used in TEM; samples were fixed using Glutaraldehyde and osmium tetroxide and embedded in epoxy resin. In the other method, liver samples were immersed in 70% ethanol, and embedded in epoxy resin. However, at observation, no clear morphological structure was seen and no Al deposition detected in all the samples. The failed attempt to detect Al in the liver

tissue might attributed to the low thickness (90 nm) of the liver sections. For the unclear morphological structure, the reason could be the absence of fixative in the liver tissue as fixative is important to preserve tissue from degradation.

Transmission electron microscopy (TEM) has also been used extensively to study cellular structure in biological materials. Due to its high spatial resolution and high magnification, transmission electron microscopy has the advantage of detecting some morphologic features in the liver at the sub-cellular levels that could not be detected using light microscopy such as loss of canalicular microvilli and swollen mitochondria (Shu et al., 1991; Cai et al., 2006). In addition, because we aimed to look at the aluminum deposition in the liver samples and where it is localized within the cell, TEM can be equipped with energy dispersive microanalysis (EDX) which has the potential to analyze all the elements in liver sample at a time. However, the weakness of electron penetration requires sample thicknesses considerably less than 1 μm (Kirz et al., 1995). Sectioning samples into thin sections can cause structural artifacts or distortion. The other challenge is the low contrast of biological materials which requires staining samples with heavy metal salts that bind to specific intracellular organelles to differentiate them which can be an another source of artifact.

Using transmission electron microscopy equipped with energy dispersive microanalysis was very effective in detecting the ultrastructural changes as well as the site of aluminum deposition in the liver. In agreement with previously published results, (Klein et al., 1987; Galle and Giudicelli, 1982; Kametani and Nagata, 2006) aluminum was found deposited in the lysosomes of the hepatocytes of the study group. Our work confirms the finding that TEM-EDX is superior tool to investigate the trace elements deposition such as aluminum in biological materials (Kametani, 2002).

The results show that daily parenteral administration of aluminum at high dose (1500 $\mu\text{g/kg/d}$) result in accumulation of hepatic aluminum associated with elevated serum total bile acids and histopathological changes in the liver. The significant elevation in serum total bile acids level was noticed as early as day 14 of aluminum administration. Comparable results were reported in neonatal piglets and rats infused parenterally with aluminum (Klein et al., 1987; Klein et al., 1988), and in rats administered intraperitoneally with aluminum (Demircan et al., 1998). This implies that daily chronic intravenous administration of aluminum induces liver injury after two weeks. In addition, elevation in serum bile acids has been seen in early onset of parenteral nutrition associated liver injury (Demircan et al., 1998; Tazuke and Teitelbaum, 2009). Our results are consistent with those reported that increased levels of bile acids are indicative of liver injury, and are in accordance with the suggested proposal by Demircan et al, (1998) that serum bile acids might be more sensitive indicator for the early diagnosis of parenteral nutrition associated liver disease (Farrell et al., 1984; Demircan et al., 1998; D'Apolito et al., 2010).

The amount of aluminum accumulated in the liver was proportionally correlated with the duration of aluminum administration (Klein et al., 1988), and was significantly correlated with the increase in total bile acids throughout the duration of the study. In our study, the effect of aluminum loading on the liver was manifested with ultrastructural changes such as loss of bile canalicular microvilli and condensation of mitochondria after two weeks of aluminum administration. On the contrary, in study conducted by Klein et al (1987), the electron microscopy observation failed to detect ultrastructural changes in the liver of piglets infused with the same dose used in our study (1500 $\mu\text{g/kg/d}$) for 50 days (Klein et al., 1987). However, in agreement with our finding, other investigators were able to demonstrate histopathological

changes in liver infused with aluminum. Stacchiotti et al., (2008) reported that in adult mice orally administered Al sulphate (2.5 %) daily for 10 months, the electron microscopy observation showed a reduction of hepatic microvillar projections by 60-80 % compared to controls. Also, Demircan et al., (1998) reported that histologic changes such as portal inflammation were detected using light microscopy in rats' liver infused intraperitoneally with aluminum.

Our data indicates that the intravenous infusion of aluminum at a high dose (1500 µg/kg/d) in growing neonatal piglets accumulate a significant amount of aluminum in serum, bile, urine, and liver tissues. Marked elevation in serum total bile acids and loss of canalicular microvilli were noticed after two weeks of aluminum administration. For detecting the site of aluminum deposition in the liver as well as study the ultrastructural morphology, we found that TEM-EDX is the best suitable technique for our purpose and it will be employed in the subsequent experiments. Also, based on elevated total serum bile acids seen after aluminum and TPN administration, we may postulate that Al loading may contribute to parenteral nutrition induced liver injury.

4.0 ALUMINUM-IRON INTERACTION AND ITS ROLE IN CAUSING LIVER INJURY IN INTRAVENOUS ALUMINUM INFUSED PIGLETS

4.1 Introduction

Aluminum toxicity is well documented, but the mechanism of action is poorly understood. Studies have demonstrated that aluminum is a potential toxicant to various body systems including central nervous system, hematopoietic system, and skeleton (Yokel and McNamara, 2001; Kausz et al., 1999; Alfrey, 1991; Perez et al., 2001; Practico et al., 2002). Because aluminum has an ionic structure similar to iron and is known to bind to transferrin, the main iron transport protein, interactions between aluminum and iron occur in a variety of cells and tissues (DeVoto and Yokel, 1994). The exact mechanism of aluminum effect on iron metabolism is not yet clear. However, Abreo et al. (1994) proposed that aluminum loading causes sequestration of iron in either the mitochondria or nuclei with the resulting reduction in cytosolic iron leading to stabilization of iron regulatory proteins (IRP) and suppression of ferritin synthesis. This could lead to increased concentrations of free iron. Alternatively, Yamanaka et al., (1999) proposed that aluminum could modify cellular iron homeostasis by stabilizing the iron regulatory protein 2 (IRP2), which would promote the binding of IRP to iron responsive elements (IRE), inducing transferrin (TfR) expression while blocking ferritin synthesis. This could lead to accumulation of intracellular free iron, and thereby increasing oxidative damage in the cell.

A number of studies conducted on a variety of cultured cell lines have shown that aluminum loading results in abnormal iron accumulation in tissues (Abreo, 1990; Abreo, 1994). In experimental animals, the increase in tissue aluminum content was paralleled by elevations of iron in liver, kidney, heart, spleen, and brain (Ward et al., 2001). This increase would imply that

the normal iron homeostasis in the cell is altered. Free iron is harmful because of its high redox activity and its potential to generate free radicals, thereby inducing cellular toxicity (Halliwell and Gutteridge, 1990; Andersson et al., 1999), and tissue damage (Crichton et al., 2002).

The ability of aluminum to promote iron-induced oxidative stress is not fully understood but it is reported that aluminium potentiates the activity of Fe ions to cause oxidative damage (Gutteridge et al., 1985; Oteiza et al., 1993; Good et al., 1992; Xie and Yokel, 1996). Bondy and Kristine (1996) observed that in the presence of aluminum salts, there is a potentiation of iron-induced reactive oxygen species (ROS), but no stimulation of ROS observed in the presence of free aluminum. Also, Abreo et al. (1990 and 1991) observed that increased iron uptake in aluminum-loaded mouse hepatocytes and Friend erythroleukemia cells were associated with membrane lipid peroxidation. This has led to the hypothesis that aluminum loading may lead to iron-induced oxidative stress, which, in turn, may explain the pathological changes associated with TPN-induced liver injury.

4.2 Hypothesis and Objective

The hypothesis of this study was that aluminum loading disrupts iron homeostasis in the liver thus resulting in increased free iron and increased oxidative stress in the liver. The objective of this study was to determine if intravenous injection of high dose aluminum into neonatal piglets disrupts iron homeostasis in the liver and whether the resulting free iron plays a role in the liver injury that is associated with aluminum exposure.

4.3 Materials and Methods

Frozen piglet liver tissues (flash frozen in liquid nitrogen and stored at -80°C) from the study (16 piglets) and control group (4 piglets) were used in this study. The study subgroups (one, two, three, and four weeks) were given daily intravenous injections of 1 mL/kg/day of

aluminum chloride hexahydrate (1 mL containing 1500 µg aluminum). The control group (4 piglets) were given daily intravenous injections of 1 mL/kg/day intravenous sodium chloride (0.9%). The study and control groups had free access to food and water (Chapter 3).

4.3.1 Liver homogenization

Prior to measuring thiobarbituric acid reactive substances (TBARS), nonheme iron, and bleomycin detectable iron, liver tissues were crushed into a fine powder under liquid nitrogen using BioPulverizer. The BioPulverizer consists of a hole made in a stainless steel base into which fits a special piston. Typically, up to 1 g of liver tissue is hard-frozen in liquid nitrogen and placed in the pre-chilled BioPulverizer, and the tissue is pulverized with a few blows to the pestle using a hammer. The powdered tissues were transferred into cryotubes and stored at -80°C for subsequent analysis.

4.3.2 Tissue measurement of oxidative stress

4.3.2.1 Thiobarbituric acid reactive substances (TBARS) assay

TBARS was measured in liver tissue as a marker of oxidative stress. The measurement of TBARS was adapted from the method of (Ohkawa et al., 1979). This method determines malondialdehyde (MDA) and other reactive aldehyde levels by heating the samples in the presence of thiobarbituric acid (TBA) at low pH, resulting in the formation of a pink chromophore with an absorption maximum near 532 nm (Abuja and Albertini, 2001). The procedure was scaled down for use with small samples (100 µL of sample), and the TBA reaction products were extracted with an equal volume of butanol/pyridine (15 butanol: 1 pyridine, v/v). Butylated hydroxytoluene (BHT), and ethylene glycol tetraacetic acid (EGTA), previously neutralized to pH 7.2, were included in the tissue homogenization buffers to prevent artefact formation of TBARS during sample preparation.

The TBARS measurement was done in duplicate for all liver tissue samples. In a micro

centrifuge tubes (1.5 ml), 50 mg of liver tissue was homogenized using a tissue grinder (Pellet Pestles® cordless motor, Kimble-Knotes # 749521-1590) with 200 µL of radioimmunoprecipitation assay (RIPA) buffer. The tissue grinder grinds soft tissues into a homogenous solution. The homogenate was incubated for 30 minutes at room temperature, and centrifuged at $15,000 \times g$ for 10 minutes at 4°C. One hundred microliters of the supernatant was added to 350 µL of thiobarbituric acid solution containing 12% acetic acid, pH 3.5, 0.6% SDS, 0.45% thiobarbituric acid, and 0.0002% BHT. The mixture was heated for 1 hour at 95°C. Then, the heated sample was cooled and centrifuged at $4,000 \times g$ for 10 min. The absorbance of the supernatant was measured at 532 nm using Beckman DU40 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). The malondialdehyde (MDA) was calculated using a standard curve of malondialdehyde prepared from tetraethoxypropane by acid hydrolysis. The amount of MDA in samples was expressed as nmol/mg of protein.

4.3.2.2 Biuret protein assay

The biuret protein assay was used to measure the protein content in tissue homogenates in order to make other measurements comparable. The method is based on the formation of a purple complex between copper salts and substances containing two or more peptide bonds in an alkaline medium. The procedure used was based on Lyne's review for the biuret method (1957). In the assay, samples were vortexed with biuret reagent (32 mM sodium potassium tartrate, 12 mM copper sulphate, 30 mM potassium iodide, 0.2 M NaOH) in a ratio of 1 sample: 9 biuret reagent (v:v), left for 20 minutes at room temperature. The copper ions form a complex with the amide groups in the proteins and create a blue color that was measured at a wavelength of 550 nm using a Beckman Coulter DU® 640B spectrophotometer. Protein concentrations of tissue homogenates were measured in duplicate by the biuret method (Layne E, 1957) using bovine serum albumin as a calibration standard.

4.3.3 Determination of free iron in liver tissues by bleomycin detectable iron

The free iron level in liver tissues was measured using the bleomycin assay method (Gutteridge et al., 1981). This method is based on the direct complexation of redox-active iron with the antibiotic bleomycin. The iron–bleomycin complex causes degradation of DNA and the degradation products can be measured. The detected iron is generally named bleomycin-detectable iron. Concentrations of bleomycin-detectable iron are believed to represent the redox-active iron pool (Gutteridge et al., 1981).

A 50 mg of liver tissue was homogenized with 500 μ L 20 mM Tris-HCl buffer pH 7.4 using a small Dounce homogenizer (an apparatus consisting of a glass tube with a tight-fitting glass pestle used manually to disrupt tissue suspensions to obtain single cells or subcellular fractions) for 1 minute. The reaction mixture was prepared by the sequential addition of the following reagents: 250 μ L DNA (1 mg/mL), 25 μ L bleomycin (Sigma, USA) (1.5 units/mL), 50 μ L of 50 mM $MgCl_2$, 50 μ L of 1 M Tris-HCl pH 7.4, 25 μ L of 1 homogenate/standard, and 50 μ L 17.5 mM ascorbic acid. The homogenate was incubated at 37°C for 1 hour (to allow the iron bleomycin complexes to bind to DNA, releasing malondialdehyde from DNA degradation). Fifty microliters of 0.1 mol/L EDTA was added to stop the reaction. Two hundred and fifty microliters of 10 g/L thiobarbituric acid and 250 μ L of 3 N HCl were added. The mixture was heated at 85°C for 20 minutes (to allow chromogen formation by the reaction of malondialdehyde with thiobarbituric acid). The mixture was cooled at room temperature, centrifuged at $10,000 \times g$ for 10 minutes. The absorbance of the supernatant was measured at 532 nm using Beckman DU40 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada).

4.3.4 Non-heme iron assay

Fifty mg of liver tissue was homogenized with 500 μ L high-purity 18 M Ω water using a Dounce homogenizer. In 1.5 mL plastic microcentrifuge tubes, 100 μ L of tissue homogenate

was added to an equal amount of 1.5 N HCl. The homogenate was heated at 85°C in heating blocks for 30 minutes, cooled in water at room temperature for 2 minutes, and centrifuged for 5 minutes at $10,000 \times g$. 100 μ L of the supernatant aliquot was transferred into another plastic centrifuge tube, and 20 μ L of 40 % trichloroacetic acid was added. The mixture was heated at 85°C in heating blocks for 15 minutes, centrifuged for 5 minutes at $10,000 \times g$ at room temperature. 200 μ L of 1 mM ferrozine solution (1 mM ferrozine in 1.05 M sodium acetate, pH 4.8) was added to 20 μ L of the supernatant aliquot. The absorbance was measured at 562 nm using spectrophotometer (Beckman DU40 spectrophotometer (Beckman Coulter, ON, Canada)).

4.4 Statistical analysis

Descriptive statistics were performed for the variables. Results were expressed as mean \pm standard deviation (SD), and analyzed using one-way ANOVA and post hoc test (Student-Newman-Keuls). A $p < 0.05$ was considered statistically significant.

4.5 Results

4.5.1 Measurement of free iron and TBARS levels in liver tissues

The concentration of bleomycin detectable iron, an indicator of the free iron pool (Guttridge, 1981), was measured in the liver tissues. There was a trend towards increased levels of bleomycin in the liver tissues of the study groups throughout the study period (Table 4.1). The bleomycin detectable iron levels in the liver tissues were proportionally increased with the duration of aluminum exposure. The increase peaked in the four week group (43%) compared to controls, and 39% compared to the one week group (Table 4.1). Also, aluminum loading caused non-significant increases in reactive aldehyde levels in all treatment groups compared to the controls (Table 4.1). The highest increase was seen in the four week treatment group (25 % increase), but levels were not significantly higher than controls.

Table 4.1. Bleomycin detectable iron and TBARS levels in piglet liver tissues (mean±SD).

Group	Bleomycin detectable iron ($\mu\text{g Fe/mg}$)	TBARS (nmol/mg)
Controls (n=4)	0.047±0.019	0.71±0.04
One week (n=4)	0.049±0.018	0.75±0.17
Two week (n=4)	0.054±0.028	0.76±0.18
Three week (n=4)	0.065±0.003	0.82±0.05
Four week (n=4)	0.068±0.014	0.87±0.07

Data are expressed as mean \pm SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($\alpha=0.05$). Bleomycin detectable iron and TBARS levels in the liver tissue were measured in all treatment groups (one week, two week, three week, and four week) after receiving daily intravenous injection of aluminum chloride hexahydrate, 1 mL/kg/day (each 1 mL contains 1500 μg of aluminum). Controls were given daily intravenous injections of 1 mL/kg/day sodium chloride (0.9%) for one week.

4.5.2 Measurement of non-heme iron levels in liver tissues

Non-heme iron levels, which consist of intracellular iron bound to ferritin, hemosiderin, and other proteins such as those containing iron-sulfur clusters in the mitochondrial electron transport chain, were measured in liver tissues. The mean levels of non-heme iron in the liver were increased in the one, two, and three week groups (Table 4.2). In the three week group, the level of non-heme iron peaked and was significantly ($p<0.05$) increased by 255% and 175%, respectively, when compared to controls and one week aluminum treatment group. In the four week aluminum treatment group, the non-heme iron levels increased by 104% compared to controls (Table 4.2).

Table 4.2. Non-heme iron ($\mu\text{g Fe/mg}$) levels in piglets liver tissues.

Group	(Mean \pm SD)
Controls (n=4)	0.45 \pm 0.28 ^a
One week (n=4)	0.58 \pm 0.04 ^a
Two week (n=4)	1.5 \pm 0.90 ^{a,b}
Three week (n=4)	1.6 \pm 1.20 ^b
Four week (n=4)	1.0 \pm 0.60 ^{a,b}

Data are expressed as mean \pm SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($\alpha=0.05$). Means with different superscripts (a, b) were significantly different ($p<0.05$) when compared to each other. Non-heme iron levels in the liver tissue were measured in all treatment groups (one week, two week, three week, and four week) after receiving daily intravenous injection of aluminum chloride hexahydrate, 1 mL/kg/day (each 1 mL contains 1500 μg of aluminum). Controls were given daily intravenous injections of 1 mL/kg/day sodium chloride (0.9%) for one week.

4.6 Discussion

In this study, we hypothesized that aluminum loading disrupts iron homeostasis in the piglet's liver thus resulting in increased free iron and increased oxidative stress in the liver tissues. The present study showed that Al loading caused significant increase in the hepatic non-heme iron levels after three weeks of Al administration. Unexpectedly, despite the increasing trend seen in levels of hepatic bleomycin detectable iron and TBARS in all the study groups, this increase was non-significant in all study groups compared to controls.

The physiological effects of aluminum have been extensively investigated, but the exact mechanism by which aluminum causes damage in the biological system is still speculative. However, one of the proposed mechanisms for Al mediated damage is believed to be through increased oxidative stress (Flora et al., 2003; Mahieu et al., 2003). Aluminum can exert its pro-oxidant effect via either formation of Al superoxide or by its ability to facilitate iron-driven biological oxidative reactions (Exley et al., 2004; Bondy et al., 1996). It has been demonstrated in experimental animals that Al exposure has a key impact on tissue iron (Fe) distribution (Ward et al., 2001). The increase in tissue Al content is associated with an increase in tissue Fe in the kidney, liver, heart, and spleen, as well as in various brain regions (Ward et al., 2001).

Aluminum has the potential to increase iron induced oxidative stress to the cell (Clauberg and Joshi, 1993; Van Rensberg et al., 1997). One suggested mechanism is that Al could induce lipid peroxidation by increasing the cellular iron uptake paralleled with decreased iron entry into ferritin (Abero and Glass, 1993), thereby increasing the free iron level and facilitating iron-induced oxidative stress. In support of this mechanism, Xie et al. (1996) reported that increased intraneuronal Al potentiated iron-induced oxidative damage and reduced neuronal survival in bovine brain.

Much research has been done on the effect of aluminum loading on iron-induced oxidative damage in various biological tissues. In mice, aluminum overload was found to be associated with lipid peroxidation (Pratico et al., 2002). In vitro studies, using liposomal and brain microsomal systems, suggest the aluminum ion has a stimulating effect on iron-induced lipid peroxidation (Oteiza et al., 1993; Quinlan et al., 1988; Verstraeten and Oteiza, 1995; Verstraeten et al., 1997). In hemodialysis patients using a phosphate binder such as aluminum hydroxide, higher lipid oxidation had been found in their plasma (Lin et al., 1996; Wratten et al., 2000; Neiva et al., 2002). Kapiotis et al. (2005) tested the influence of Al on low-density lipoprotein (LDL) oxidation by Fe ions and Cu ions. The results showed that aluminum ions increased the metal ion-induced oxidation of LDL in the presence of Fe ions, but not Cu ions (Kapiotis et al., 2005).

The effect of Al and its role in causing iron-induced oxidative stress is well-documented (Harley et al., 1993; Jang and Surth 2002; Walter et al., 2002; Pratico et al., 2002). However, in our study, even though Al loading in serum and liver tissues was significant (section 3), the data did not statistically show a significant increase in the levels of free iron and lipid peroxidation in all the study groups compared to controls. Nevertheless, the increasing trend seen in the hepatic levels of free iron and lipid peroxidation may reflect the potential of Al to play an important role in causing increased free iron and oxidative damage via its pro-oxidative effect.

At this time, using our data with a low n of 4, we cannot confirm that Al alters cellular iron homeostasis in the liver tissue and plays a role in causing oxidative stress via its pro-oxidant effect on iron.

During the study, a number of important limitations need to be considered. First, the total iron concentration was not analyzed in the serum and liver tissue of the piglets. Knowing the total iron content of serum and liver tissues would help to understand the relationship between Al loading and iron content in the piglet's serum and liver tissue. The decision to study the effect of Al on iron homeostasis in the liver tissues was made after the serum and liver samples were already analyzed for Al at the Saskatchewan Research Council. Further analyses of total iron were not done due to limited samples and the high cost of sample analysis using the inductively coupled plasma spectrometry (ICP-MS). Second, the sample size used in the study is small. We had only four animals in each of the five groups. It is known that sample size is an important part of the study, and larger samples tend to minimize the probability of type II errors, maximize the accuracy of study estimates, and increase the generalizability of the results obtained in the study.

5.0 INVESTIGATING THE EFFECT OF LOW ALUMINUM CONTENT IN PARENTERAL NUTRITION ON THE DEVELOPMENT OF PARENTERAL NUTRITION ASSOCIATED CHOLESTASIS IN NEONATAL PIGLETS

5.1 Introduction

Aluminum is the third most abundant metal in our environment. Aluminum is a known contaminant of parenteral nutrition solutions and is suspected in the pathogenesis of parenteral nutrition associated cholestasis (PNAC). The PN components that have the highest amounts of Al include calcium gluconate, potassium phosphate, and sodium phosphate (Bohrer et al., 2002; Smith et al., 2007). Calcium gluconate alone attributes > 81% of the total aluminum content in PN solutions (Mouser et al., 1998). Aluminum is found not only in raw materials but also is incorporated into products during the manufacturing process and from the leaching of aluminum from glass containers during autoclaving for sterilization (Bohrer et al., 2002).

Physiologically, the body has several mechanisms to help reduce the absorption of aluminum. The gastrointestinal tract (GIT) barrier allows only < 1% of ingested aluminum into the blood circulation (Sedman et al., 1985). The major route of aluminum elimination is via renal excretion with minor excretion in bile (Sutherland and Greger, 1998). Consequently, when parenterally infused significant aluminum accumulation can occur in neonates, in particular premature neonates who have immature renal elimination mechanisms as well as the need for more calcium salts for bone mineralization. The aluminum content in the PN solutions delivered to patients in the neonatal intensive care unit may range from 10 – 60 µg/kg/day (Klein et al., 1991; Mouser et al., 1998; Popinska et al., 1999; Advenier et al., 2003). This significantly exceeds the American Society of Clinical Nutrition/American Society of Enteral and Parenteral Nutrition Joint Commission (ASCN/ASPEN 1991) recommendation of 2.0 µg/kg/day, as a safe

level for aluminum intake in adults. Also, it exceeds the safe limit of 5 µg/kg/day Al recommended by the Food and Drug Administration and Health Canada (FDA, 2000; Health Canada, 2011). However, for premature neonates with very immature renal elimination mechanisms, levels of 2 µg/kg/day may still pose a significant health risk.

Although research has suggested a correlation between aluminum loading and PNAC in neonates, the exact role of aluminum in the development of PNAC is unknown. A previous graduate student in our lab found that 31% of infants with gastrointestinal failure who received parenteral nutrition (PN) for 21 days developed cholestasis (Arnold et al., 2002). Although these PN solutions were contaminated with 20 µg/kg/d aluminum (compared to the recommended 'safe' level of 2 µg/kg/d for adults), its causal relationship to PNAC remains speculative. In a neonatal piglet model, infusion of PN solutions with 38 µg/kg/d aluminum for 3 weeks led to early cholestasis; but a low aluminum PN was not tested. Also, infusion of a daily bolus of 1500 µg/d aluminum in orally fed neonatal piglets has led to cholestasis, but not 20 µg/d, for 3 weeks. Although this bolus regimen is less relevant to the PN-fed neonate, it is being used to explore more sophisticated imaging techniques in an attempt to identify more subtle histological changes in early stages of cholestasis. In this study, we have used the more clinically relevant TPN-fed piglet model and evaluated cholestasis markers after infusing high or low aluminum PN. This study was conducted in Dr Bertolo's lab, Memorial University of Newfoundland. The collaboration with Dr. Bertolo leverages his extensive experience in studying PN therapy and expands his field of research. The Yucatan miniature pig is smaller and slow growing than domestic pigs; the slower growth is more appropriate as a model for infants and allows for prolonged PN studies, as well as being easy to care for. Dr. Bertolo's laboratory has extensive experience administering PN using this model and he is able to handle a large number of subjects

at one time providing the opportunity to complete the proposed study in an efficient manner. Considering the substantial vulnerability of neonates to Al toxicity, both because of high Al exposure from PN and potentially impaired Al elimination, more research needs to be done to investigate the exact role of Al in causing parenteral nutrition associated liver injury.

5.2 Hypothesis and Objective

The hypothesis for this study is that reduction in the aluminum content of PN solutions causes significant reductions in the incidence and severity of liver injury in neonatal piglets. The main objective for this study was to investigate the role of aluminum as a toxic component of parenteral nutrition and as a risk factor in causing liver injury. The present study utilizes a combination of biochemical and morphological methods in order to test our hypothesis.

We have evaluated the effects of high and low aluminum levels in PN by measuring several biochemical and histologic parameters of liver injury including serum direct bilirubin levels, serum total bile acid levels, and morphological changes in the liver tissues. Also, for aluminum loading, we measured the aluminum content in the liver, serum, and bile.

5.3 Material and Methods

The Institutional Animal Care Committee at Memorial University of Newfoundland approved all animal procedures and protocols used in this study, and all animals were treated in accordance with guidelines set by the Canadian Council on Animal Care.

Sixteen Yucatan miniature pigs were obtained from a breeding herd at Memorial University of Newfoundland, St John's, NL. Piglets were taken from sows at 2 – 4 days of age and randomly assigned into two equal groups:

(1) Regular PN group, which consisted of 8 animals. In this group, piglets received continuous PN infusion with a standard PN formulation to meet all nutritional requirements (NRC, 1998)

with aluminum contamination at 38 µg/kg/ day.

(2) Low aluminum PN group, which consisted of 8 animals. The piglets in this group were treated identically to regular PN group but with the PN solution modified by substituting the standard calcium gluconate additive for an ultrapure form (Sigma Aldrich, Oakville, Canada) that reduced the aluminum contamination to 6 µg/kg/day.

5.3.1 Surgery

Piglets at the age of 2-4 days were removed from the sow and transported to the animal housing facility in the Biotechnology Center, Memorial University of Newfoundland. Upon arrival, the piglets of both low aluminum PN (n=8) and regular PN (n=8) groups immediately underwent surgical implantation of two central venous catheters. All the procedures were carried out under general anesthesia in a certified operative facility at the Biotechnology Center, Memorial University of Newfoundland. Prior to the induction of anesthesia, piglets were pre-anesthetised with an intramuscular injection of ketamine hydrochloride (0.22 mg/kg, Bimeda Canada, Cambridge, Ontario) and acepromizine (0.5 mg/kg, Vetoquinol, Canada, Inc, Quebec, Canada). Anaesthesia was maintained with 1.5 – 2 % isoflurane delivered with oxygen at 1.5 L/min via an endotracheal tube. Under general anaesthesia, an incision of approximately 2 centimeters long was made on the right side of the neck. An autoclaved silastic catheter (Dow Corning Co, Midland, USA; 0.27 inch ID, 0.63 inch OD) was introduced into the external jugular vein and advanced to the superior vena cava, just cranial to the heart, to be used for diet infusion. A second catheter (Dow Corning Co, Midland, USA; 0.04 inch ID, 0.08 inch OD), used for blood sampling, was introduced into the left femoral vein and advanced to the inferior vena cava just caudal to the heart. Both catheters were tunneled under the skin and exited at the back, dorsally between the piglet's shoulder blades. Both ends of the catheters were fitted under

the bandage on the back of the piglet. Immediately following surgery, all animals were given intravenous doses of antibiotic 0.5 mL of Borgal (40 mg trimethoprim & 200 mg sulfadoxine/mL) (Intervet Canada Ltd.,) and analgesic (0.03 mg/kg buprenorphine hydrochloride) (Temgesic, Reckitt Benckiser Healthcare (UK) Ltd.,). The neck incisions were closed with 3-0 Novafil suture and the incisions sites were treated with a topical antibiotic until healed. The piglets were fitted with elastic bandages to protect the catheter. The catheter was flushed with one mL of 0.9% sodium chloride solution and then one mL of heparinised 50% dextrose to maintain the patency of the catheter after drawing blood.

5.3.2 Care of animals

Piglets were housed in a Canadian Council on Animal Care certified animal care facility at Memorial University of Newfoundland in individual circular cages that allow visual and aural contact with one another and they had a toy for environmental enrichment. The cages were controlled with a 12 hr light/dark cycle, and ambient temperature was kept at 27°C, with supplemental heat provided by heat lamps. Piglets were fitted with cotton jackets that secured the catheters to a swivel and tether system. This system allows for the continuous infusion of parenteral fluids while allowing the piglet to move freely about the cage without twisting or tangling the tubing. Hospital-grade, pressure sensitive PN infusion pumps were used to provide a continuous, PN infusion. On the day of surgery (day 0), piglets were adapted to a parenteral diet infusion that was initiated immediately after surgery at 50% and slowly advanced over 24 hours to the goal infusion rate. Piglets received nothing by mouth and were monitored daily for PN intake, weight gain, body temperature, feces and urine production. Cages were cleaned daily. The piglets were having access to a toy for entertainment, and a blanket for resting.

5.3.3 Parenteral nutrition administration

The PN formulation was designed to provide all nutrients required by the neonatal piglets. The intravenous nutrient requirement for piglets has been estimated from orally fed piglets (NRC, 1998). In total, piglets received 1.1 MJ/kg/d, approximately 260 kcal/kg/d and 200 kcal/kg/d were from non-protein sources. PN solutions were prepared daily using aseptic technique. Lipid emulsion (Intralipid 20%; Fresenius-Kabi, Germany) and PN solutions were mixed and delivered at a rate of 320 mL/kg/d by infusion pumps for 14 days. All of the PN component solutions used in the PN regimen were the same as commercial products used in hospitals for infants. The amount of aluminum content in these PN solutions was measured by inductively coupled plasma-mass spectroscopy (ICP-MS) at SRC, Saskatoon, Saskatchewan, Canada.

5.3.4 Sample collection and analysis

Daily weight was recorded. Blood samples (5 mL) were drawn by syringe from the femoral access catheter into metal-free vacutainer tubes from piglets at baseline (prior to PN administration) and at day 7, and 14. The blood was centrifuged (Clinical 200, VWR, Canada) at 5000 rpm for 5 minutes, and serum was aliquoted into three parts. The aliquots were each used to measure the following: 1) direct bilirubin using a bilirubin assay kit (Bioassay Systems, Hayward, CA, USA) and spectrophotometric analysis according to manufacturer instructions, 2) total bile acids using a bile acid assay kit (BioQuant, San Diego, CA, USA) and spectrophotometric analysis according to manufacturer instructions, and 3) aluminum concentration using inductively coupled plasma mass spectrometry (ICP-MS) at the Saskatchewan Research Council (SRC), Saskatoon, SK. Piglets were killed humanely on day 14 of the study. Bile from gallbladder was collected using syringe needles and liver samples were quickly collected in metal free plastic containers and sent to SRC for aluminum determinations

by inductively coupled plasma mass spectrometry (ICP-MS). For histopathological observation, liver sections were cut and processed for light microscopy, transmission electron microscopy (TEM), and scanning transmission x-ray microscopy (STXM) as indicated previously in section 2. The remaining liver tissues were snap frozen and stored at -80°C for further studies.

5.3.5 Microscopic and imaging techniques sample preparation

5.3.5.1 Light microscopy

Liver tissues were processed and prepared (as mentioned previously) in the Department of Anatomy and Cell Biology, University of Saskatchewan. Sections were stained for routine light microscopy with hematoxylin and eosin (H&E) staining, cover slipped and sent to the Western College of Veterinary Medicine, University of Saskatchewan, SK, to be evaluated blindly by a veterinary pathologist for histological changes. Samples were evaluated using a scoring system as follow (see Table 5.1) (Loff et al., 1998; Cai et al., 2006).

Table 0.1. Histologic scoring system for histologic changes in the piglet liver.

Score	Histologic changes
0	Normal modality without degeneration
1	Mild to moderate inflammation
2	Moderate degenerative changes
3	Cholestasis or mild fibrosis

5.3.5.2 Transmission electron microscopy (TEM)

The fixation and embedding of liver samples was done in the Faculty of Science, Memorial University of Newfoundland. The liver samples were processed as follows: liver

sections were cut into no larger than 1mm³ pieces with a sharp razor blade. The sections were fixed primarily with 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) by immersing the liver pieces in the glutaraldehyde fixative and kept in the refrigerator for 4 hours. The liver sections were washed with the sodium phosphate buffer three times for 30 minutes each. Then, the samples were fixed (secondary fixation) with 4 % osmium tetroxide in 0.1 M sodium phosphate buffer by immersing the liver pieces in the osmium tetroxide fixative and kept in the refrigerator for 4 hours. The samples were washed again with cold sodium phosphate buffer for 15 minutes and were dehydrated in a graded series of ethanol (25%, 50% 70-75%, 90-95%, 100%) for 10 minutes each. Then, the samples were washed in distilled water on ice for 30 minutes. The samples were infiltrated with the following ratios of epoxy resin: propylene oxide (1:2, 1:1, 2:1, 1:0) simultaneously for one hour each. The samples were embedded in 100% epoxy resin in small plastic capped vials. The plastic vials were placed in oven at 70°C for more than 8 hours until the resin was completely polymerized. Samples were sent to the Pathology Department, College of Veterinary Medicine, University of Saskatchewan to be cut and mounted on copper grids. For histopathological observation, the liver samples were observed using a transmission electron microscope at the Department of Biology, University of Saskatchewan. Electron micrograph images for the liver cellular ultrastructure were taken. Films were developed and scanned in the Department of Biology, University of Saskatchewan. However, for canalicular microvilli morphology, a grading system for the changes in the bile duct microvilli was created to compare the morphological changes in the bile canalicular microvilli (Table 5.2) and the liver samples were evaluated blindly by an expert (PhD Anatomy & Cell Biology) in liver pathology.

Table 0.2. Morphology scoring of bile canalicular microvilli.

Score	Microvilli morphology
0	Normal
1	Short microvilli
2	Total loss of microvilli

5.4 Statistical analyses

Descriptive statistics were performed for the variables. Results were expressed as mean \pm standard deviation (SD), and analyzed using one-way ANOVA and Post Hoc (Student-Newman-Keuls test). For non-parametric data, Mann-Whitney U test was used for the analysis. A p value < 0.05 was considered significant.

5.5 Results

5.5.1 Body weight

Mean body weight gain data is summarized in Table 5.3. At the end of the study, the mean body weight in the low aluminum group was higher than the regular PN group, but this difference was not statistically different.

Table 0.3. Body weight gain (Mean \pm SD) and growth rate of piglets during the experimental period.

Group	Initial body weight	Final body weight	% Growth rate
Low Aluminum PN (n=8)	1832 \pm 338	3461 \pm 468	4.6
Regular PN (n=8)	1728 \pm 282	3312 \pm 530	4.7

Data were expressed as mean \pm SD

5.5.2 Biochemical assays

5.5.2.1 Serum direct bilirubin and total bile acid

The mean serum direct bilirubin level was more elevated in the regular PN group in day 0 and day 14 compared to the low aluminum PN group (Table 5.4). However, the difference in serum direct bilirubin levels between regular and low aluminum PN groups was not significant.

The mean total bile acids in the regular PN group were higher at day 7 and 14 compared to the low aluminum PN group (Table 5.5). Despite the marked elevation in the level of serum total bile acids in the regular PN group compared to low aluminum PN group, the difference was not statistically significant.

Table 0.4. The mean concentration of serum direct bilirubin in piglets during the study.

Group	Direct bilirubin ($\mu\text{mol/L}$)		
	Day 0	Day 7	Day 14
Low Aluminum PN	8.8 \pm 4.9	17.3 \pm 6.4	25.9 \pm 10.0
n	7 ¹	8	8
Regular PN	9.7 \pm 3.6	17.0 \pm 9.6	29.0 \pm 9.3
n	8	8	8

Data were expressed as mean \pm SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($\alpha=0.05$).

1. Low aluminum group had one sample with insufficient serum at day 0 (piglet # 25).

Table 5.5. The mean concentration of serum total bile acids in piglets during the study.

Group	Total Bile acids ($\mu\text{mol/L}$)		
	Day 0	Day 7	Day 14
Low Aluminum PN	45 \pm 62	56 \pm 58	148 \pm 71
n	8	6 ¹	8
Regular PN	29 \pm 20	61 \pm 40	198 \pm 75
n	7 ²	8	8

Data were expressed as mean \pm SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($\alpha=0.05$).

1. Low aluminum group had two insufficient samples for analysis on day 7 (piglet # 2 & 22).
2. Regular PN group had one insufficient sample for analysis on day 0 (piglet # 24)

5.5.2.2 Aluminum concentration in serum, liver, and bile

There was no difference in the mean serum aluminum concentrations between the regular PN and low aluminum group on day 0 and 7 (Table 5.6). On day 14, the mean serum aluminum level was significantly ($p<0.05$) higher in the regular PN group than the low aluminum group (Table 5.6). The mean hepatic aluminum content in the low aluminum PN group was significantly ($p<0.05$) lower compared to the regular PN group (Table 5.7). The mean bile aluminum concentration was lower in the low aluminum PN group compared to the regular PN group (Table 5.7), but this was not statistically significant.

Table 0.6. The concentration of serum aluminum in piglets during the study.

Group	Aluminum (µg/L)			
		Day 0	Day 7	Day 14
Low Aluminum PN	mean±SD	320±261	504±484	218±185 ^a
	n	6 ¹	7 ²	7 ²
Regular PN	mean±SD	181±119	925±1631	884±630 ^b
	n	7 ³	7 ³	7 ³

Data were expressed as mean ±SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($p < 0.05$). Means with different Superscripts (a, b) were significantly different ($p < 0.05$) when compared to each.

1. Low aluminum PN group had two samples lost (piglet # 10, 28) for day 0.
2. Sample with insufficient serum (piglet # 28, day 7 and piglet # 25, day 14)
3. Regular PN group had three samples lost during lab relocation (piglet # 15) for day 0,7,14.

Table 0.7. Liver and bile content of aluminum in the piglets.

Group	Liver (µg/g)	Bile (µg/L)
Low Aluminum PN	0.30±0.20 ^a	218±86
n	6 ¹	6 ²
Regular PN	0.77±0.18 ^b	250±131
n	8	7 ³

Data were expressed as mean ±SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($\alpha = 0.05$). Means with different superscripts (a, b) were significantly different ($p < 0.05$) when compared to each.

1. Low aluminum PN group had one sample missed (piglet # 6) and one sample insufficient for analysis (piglets # 22).
2. One bile sample was insufficient for analysis (piglet # 25), and one sample (piglet # 2; 7200 µg/L) was considered as outlier.
3. One bile sample was considered as outlier (piglet # 8; 3600 µg/L).

5.5.3 Histopathological observations

5.5.3.1 Light microscopy

Liver tissues of low aluminum PN and regular PN groups were examined for histological changes and the morphological changes seen were given scores (see Table 5.1). Morphological evidence of mild to moderate degrees of inflammation was seen in all groups. Mild to moderate inflammation were seen in only one subject in the low aluminum PN, and in 3 subjects in the regular PN group (Table 5.8). More significant ($p < 0.012$) histologic changes were seen in the regular PN group compared to low aluminum PN group (Table 5.8).

Table 0.8. Comparison of histologic scoring for morphological changes in piglet liver.

Group	Score			
	0	1	2	3
Low Aluminum PN (n=7) ¹	6	1	0	0
Regular PN (n=8)	2	3	1	2

The differences is significant between the two groups, $p < 0.05$ ($P = 0.012$) by Mann-Whitney U test.

1. Low aluminum PN group had one missing sample (piglet # 10)

5.5.3.2 Transmission electron microscopy (TEM)

Transmission electron microscopy was used to examine the morphological changes in the liver samples. Evidence of loss of bile canalicular microvilli and mitochondria condensation were observed in most subjects in the regular PN group. There is a marked loss of canalicular microvilli seen in the regular PN group compared to low aluminum PN group (Figure 5.1). The

loss of bile canalicular microvilli was significantly different in the regular PN group from the low aluminum PN (Table 5.9).

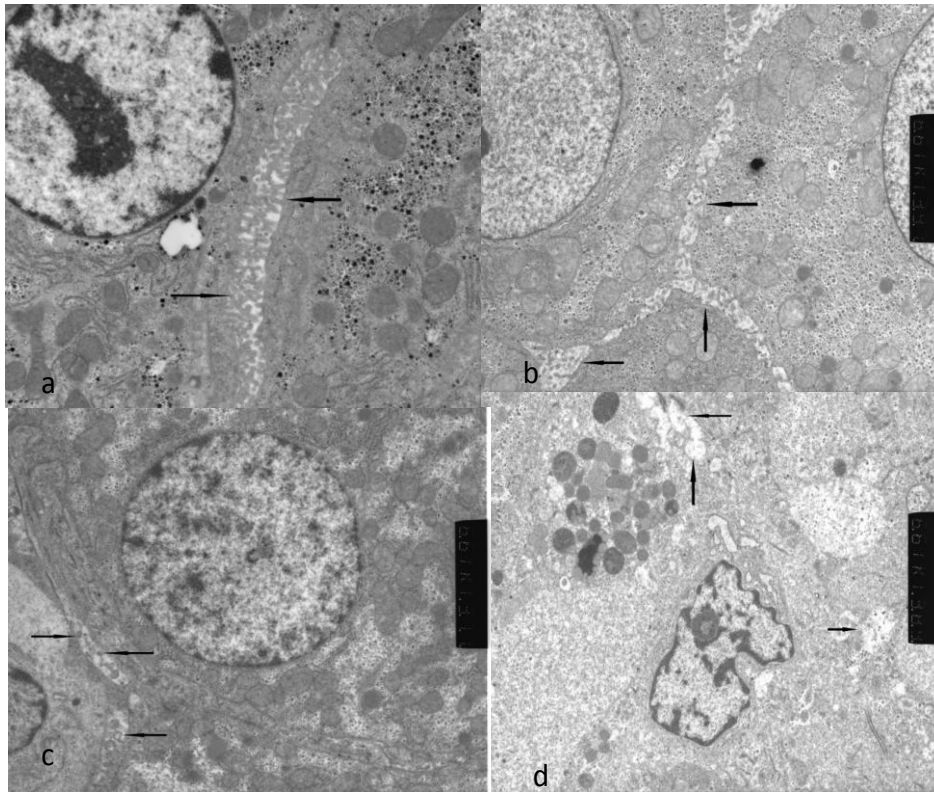


Figure 5.1. Electron micrographs showing the bile microvilli on piglet liver.

a) Microvilli in controls, x 15000 (b) normal microvilli from low aluminum PN group, x 6600 (c) short bile microvilli from regular PN group, x 6600 (d) total loss of bile microvilli from regular PN group, x 6600.

Table 5.9. Comparison of bile canalicular microvilli changes scoring.

Group	Score ^a		
	0	1	2
Low aluminum PN (n=6) ¹	4	2	0
Regular PN (n=7) ²	0	3	4

The difference between the two groups was analyzed using Mann-Whitney U-test.

The test was significant ($p = 0.006$).

1. Low aluminum PN had two samples (sample # 10 and 28) were not included in analysis; sample # 10 was lost and # 28 was poorly prepared.

2. Regular PN group had one sample excluded (sample # 24). The tissue was damaged and morphological structure of the liver was not clear.

a. Microvilli morphology score: 0, normal microvilli; 1, short microvilli; and 2, total loss of microvilli.

5.6 Discussion

This study demonstrates that reducing the aluminum content of PN decreases the degree of PN-associated liver injury. The severity of canalicular microvilli damage is related to the aluminum content of PN infused. A decrease in size and number of canalicular microvilli is a common component of the ultrastructural pathology observed in cholestasis (Phillips et al., 1987; Dancygier and Schirmacher, 2010). These microvilli are the location of bile acid transporter proteins, such as bile salt exporter pump (Bsep) and multidrug resistance-associated protein 2 (Mrp2), which play important roles in bile secretion. We suspect that the microvilli damage causes a loss of bile acid transporter proteins and a reduction in bile flow. In a study of rats administered intraperitoneal aluminum for 3 months, investigators found a loss of structural integrity of the canalicular microvilli, decreased Mrp2 content, and decreased bile flow (Gonzalez et al., 2007; Klassen, 2002).

These transporters are important in bile acid metabolism under normal circumstances. Premature infants though, may have abnormalities in any or all of the various components of bile acid metabolism, including conjugation, uptake, secretion, and recirculation (Carter and Shulman, 2007). The challenges of bile acid metabolism for premature infants may be made even more difficult with the loss of bile acid transporters secondary to the microvilli damage. Furthermore, they may not be able to up-regulate gene expression to meet the increased demand for these proteins. Variations in the developmental profiles of bile acid transporters among infants of varying developments might explain why one infant develops severe PNAC, whereas another infant with similar gestational age and feeding history has much less severe PNAC.

Serum direct bilirubin and total bile acids are early biochemical markers for PN-associated liver injury. They rise steadily over the course of therapy. However, we did not find a correlation between the levels of these biochemical markers and the amount of aluminum

loading. This is in contrast to studies of intraperitoneal aluminum injection in rats where a significant increase in plasma bile salts has been shown (Gonzalez et al., 2007; Klein et al., 1988). These subjects, however, were exposed to a much longer duration of aluminum loading. In a newborn piglet model administered parenteral aluminum for 4 weeks, we have demonstrated a correlation between aluminum loading and rising total bile acid levels (Alemmari et al., 2011). The duration of PN therapy is a risk factor for PNAC. After 6 weeks of PN therapy, 30% of children will have signs of liver dysfunction, and this increases to 67% at 16 weeks (Suita et al., 1982). It may be that the difference in biochemical markers between our study groups may have reached statistical significance with a longer duration of PN therapy.

The significant difference in the severity of microvilli damage between groups may indicate that the loss of structural integrity is an early sign of PNAC and precedes the biochemical changes. It is noteworthy that the onset of neonatal PNAC after starting PN varies with the presence or absence of risk factors. The commonly recognized risk factors for PN-associated liver injury are as follows: prematurity, duration of PN therapy, lack of enteral feeding, repeated bouts of infection or sepsis, and various toxicities or deficiencies in the PN solution. In our model, the piglets did not have the risk factors of prematurity and sepsis. The absence of prematurity and sepsis and a longer duration of therapy may have affected the differences in biochemical markers between study groups that could have reached statistical significance. Also, the small number of subjects may have limited the power of the study. Similar to our previous study of aluminum loading, we found that the serum aluminum levels ranged widely throughout the study in all piglets (Alemmari et al., 2011). Unbound aluminum in the blood is dependent on the renal glomerular filtration rate for excretion. Aluminum bound to proteins such as transferrin is nonfilterable. Variability in the functional maturity of the renal

system in newborn pigs may be a confounding variable. Other sources of parenteral aluminum, such as other pharmaceutical products, cannot be excluded. In addition, aluminum is ubiquitous in nature, and blood specimens are prone to contamination in collection, storage, pre-treatment, and analysis (Valkonen et al., 1997).

For the analysis of the microvilli damage, we used a blinded observer and a semiquantitative technique. To further quantify the degree of microvilli damage, future studies will measure the content of cytoskeletal proteins such as radixin and villin. These proteins are important in linking actin to the plasma membrane. Using proven immunohistochemistry techniques (Kojima et al., 2003; Phillips et al., 2003), we may be able to measure differences in radixin and villin activity and, in this way, provide more objective evidence to support our finding that reducing aluminum contamination of PN reduces the damage to canalicular microvilli.

The problem of aluminum contamination in PN therapy is well known (Poole et al., 2008; Klein et al., 1982; Sedman et al., 1985; deVernejoul et al., 1985). The American Society for Clinical Nutrition has recommended that the degree of aluminum contaminating adult PN therapy should not exceed 2 µg/kg/day (ASCN/ASPEN). The US Food and Drug Administration warn that levels in excess of 5 µg/kg/day are potentially toxic (FDA). Since 2004, they have required large-volume parenterals to limit aluminum contamination to no more than 25 µg/L.

However, there are no such limits for small-volume parenterals, and these are the main source of aluminum in PN. As a result, a typical infant PN therapy results in aluminum contamination levels of 10 to 60 µg/kg/day (Arnold et al., 2004; Poole et al., 2008; Moreno et al., 1994; Demircan et al., 1998). This is well in excess of the toxicity warnings of the Food and Drug Administration. This may be caused by the relatively larger amounts of calcium gluconate

that are required for growing infants compared with adults. In excess of 80% of the aluminum in infant PN therapy comes from the calcium gluconate component of the solution (Mouser et al., 1998). Infants weighing less than 3 kg on PN therapy receive the largest daily doses of aluminum (Poole et al., 2008). Also, infants may have a decreased capacity to eliminate aluminum. The elimination of aluminum is primarily dependent on urine excretion. Many premature infants have immature renal systems and may not have sufficient glomerular filtration to eliminate aluminum loads. Parenteral nutrition–associated cholestasis is a multifactorial problem. For this reason, it will require multiple strategies to both prevent and treat this problem. We propose that this study provides further evidence of the hepatotoxic effect of parenteral aluminum and that reducing the aluminum contamination of PN therapy is one important strategy in preventing or reducing the severity of PNAC.

Finally, two important limitations need to be considered in this study. The small number of piglets used in this study. A larger sample would provide a better statistical power and produce estimates that are more precise. Also, because of the lack of instruments needed (inductively coupled plasma-mass spectrometry) to analyze Al content in serum and liver tissues in St John's, NL, samples had to be shipped from there to our lab in Saskatoon. A better setup of lab equipped with the necessary instruments and equipments to conduct research would save time, money, and more importantly minimize any problems may emerge because of handling and storage during the shipping of the frozen samples.

6.0 DISPLACEMENT OF IRON AND ASSOCIATED OXIDATIVE STRESS IN THE HEPATOTOXICITY OF ALUMINUM-CONTAINING PARENTERAL SOLUTIONS DELIVERED TO PIGLETS

6.1 Introduction

Parenteral nutrition associated cholestasis (PNAC) is primarily a pediatric disease with the smallest and most premature infants being particularly vulnerable. It is estimated that 40-60% of infants on long-term parenteral nutrition (PN) for intestinal failure develop PNAC (Kelly, 2006). Of those infants born at < 1 kg, overall 23% developed PNAC, and with prolonged therapy, 80% and 90% developed PNAC after 60 and 90 days, respectively.

Over the past 30 years, there has been very little progress in the development of preventative or therapeutic strategies for treating PNAC. The complexities of PNAC is impacted by numerous factors such as prematurity, sepsis, lack of enteral feeding, and toxic or deficient components of the infused solutions. As a result, there has been a great deal of research to understand how PN therapy can cause PNAC.

One area of investigation has been an evaluation of the components of the PN solution that may be toxic to the liver (hepatotoxic). Aluminum is a non-essential element that contaminates PN solutions, and is suspected in the pathogenesis of PNAC. Most of the components of PN currently used are contaminated with aluminum with the greatest contamination occurring in calcium and phosphate solutions, as well as dextrose and trace element preparations (Greger and Sutherland, 1997; Davis et al., 1999; Li et al., 2004). Once aluminum (Al) is absorbed, it can either remain free (aluminum ions), form complex with organic acids or bound to the plasma protein transferrin, which is thought to be the mechanism by which Al is delivered to the liver (Klein et al., 1993, DeVoto and Yokel, 1994). Thus, Al could interfere with uptake,

homeostasis, and transport of iron. In cultured glial cells Al has been shown to increase the uptake of non-transferrin-bound and transferrin bound iron (Fe), likely by an effect on cellular transporters (Kim et al., 2007).

Ferritin is a ubiquitous intracellular protein that stores iron and releases it in a controlled fashion. In cultured erythroleukemia cells, Al has been shown to increase the uptake and cellular iron content while decreasing ferritin (Abreo et al., 1994). In experimental animals, Al exposure has an impact on tissue iron distribution; the increase in tissue Al content was paralleled by elevations of tissue iron in the liver, kidney, heart, and spleen, brain tissues (Ward et al., 2001, Turgut et al., 2004). Another study in chicks however, found that dietary Al decreased liver total iron but also decreased ferritin, resulting in increased non-heme iron to ferritin ratios (Han and Dunn, 2000). Interactions between Al and iron can theoretically also occur in cells and tissues due to an interaction of Al with ferritin (Fleming and Joshi 1991, Sakamoto et al., 2004; Kim et al., 2007). As a result, free iron may accumulate in body tissues. Free Fe is harmful because of its high redox activity and its potential to generate free radicals, thereby inducing cellular toxicity (Halliwell and Gutteridge, 1990; Andersson et al., 1999) and tissue damage (Crichton et al., 2002).

6.2 Hypothesis and objectives

We hypothesize that contamination of parenteral solutions with aluminum disrupts iron homeostasis in the liver resulting in an increased free iron pool, thus increasing oxidative stress and liver injury in neonatal piglets. The main objectives of this study were to evaluate the effect of reducing the aluminum contamination of parenteral nutrition on liver iron homeostasis and to determine if reducing the aluminum content of PN would reduce the free iron pool in the liver and reduce the signs of liver injury in newborn piglets.

6.3 Materials and methods

Frozen liver tissues from piglets of both low aluminum PN and regular PN groups were used in this study. The piglets (n=8) from the regular PN group received PN solutions with aluminum contamination at 38 $\mu\text{g/kg/day}$ for 14 days, and piglets (n=8) from the low Al PN groups received PN with reduced aluminum contamination to 6 $\mu\text{g/kg/day}$ for 14 days. The liver tissues were used for TBARS, non-heme iron, and bleomycin detectable iron assays. Aluminum content in liver, serum, and bile and iron content in liver and serum was assayed using (ICP-MS).

6.3.1 Thiobarbituric acid reactive substances (TBARS) assay

The measurement was done in duplicate for all liver tissue samples, using a method adapted from Ohkawa et al. (1979). In microcentrifuge tubes (1.5 ml), 50 mg of liver tissue was homogenized using a tissue grinder (Pellet Pestles® cordless motor, Kimble-Knotes # 749521-1590) with 200 μL of radioimmunoprecipitation assay (RIPA) buffer. The tissue grinder grinds soft tissues into a homogenous solution. The homogenate was stood for 30 minutes at room temperature, and centrifuged at $15,000 \times g$ for 10 minutes at 4°C . 100 μL of the supernatant was added to 350 μL of thiobarbituric acid solution containing 12% acetic acid, pH 3.5, 0.6% SDS, 0.45% thiobarbituric acid, and 0.0002% BHT. The mixture was heated for 1 hour at 95°C . Then, the heated sample was cooled and centrifuged at $4,000 \times g$ for 10 minutes. The absorbance of the supernatant was measured at 532 nm using a Beckman DU40 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). The malondialdehyde (MDA) was calculated using a standard curve of malondialdehyde prepared from tetraethoxypropane by acid hydrolysis. The amount of MDA in samples was expressed as nmol/mg of protein.

6.3.2 Biuret protein assay

Biuret protein assay was used to measure the protein concentration in tissue homogenates in order to make other measurements comparable. In the biuret assay, protein samples were combined with biuret reagent (32 mM sodium potassium tartrate, 12 mM copper sulphate, 30 mM potassium iodide, 0.2 M NaOH). The copper ions form a complex with the amide groups in the proteins and create a blue color that was measured at 550 nm using a Beckman Coulter DU® 640B spectrophotometer. Protein concentrations of tissue homogenates were measured in duplicate by the biuret method (Layne, 1957) using bovine serum albumin as a calibration standard.

6.3.3 Determination of free iron in liver tissues by bleomycin detectable iron

About 50 mg of liver tissue was homogenized with 500 µL 20 mM Tris-HCl buffer, pH 7.4 using small Dounce homogenizer for one minute. The reaction mixture was prepared by the sequential addition of the following reagents: 250 µL DNA (1 mg/mL), 25 µL bleomycin (Sigma, USA) (1.5 units/mL), 50 µL 50 mM MgCl₂, 50 µL 1 M Tris-HCl, pH 7.4, 25 µL 1 homogenate/standard, and 50 µL 1 7.5 mM ascorbic acid. The homogenate was incubated at 37°C for 1 hour (to allow the iron bleomycin complexes to bind to DNA, causing reactive aldehyde formation). Then, 50 µL of 0.1 mol/L EDTA was added to stop the reaction and 250 µL of 10 g/L thiobarbituric acid and 250 µL of 3 N HCl were added. The mixture was heated at 85°C for 20 minutes (to allow chromogen formation by the reaction of malondialdehyde with thiobarbituric acid). The mixture was cooled at room temperature, and centrifuged at $10,000 \times g$ for 10 minutes. The absorbance of the supernatant was measured at 532 nm using Beckman DU40 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada).

6.3.4 Non-heme iron assay

Fifty mg of liver tissue was homogenized with 500 μ L high-purity 18 M Ω water using a Dounce homogenizer. In 1.5 mL plastic microcentrifuge tubes, 100 μ L of tissue homogenate was added to an equal amount of 1.5 N HCl. The homogenate was heated at 85°C in heating blocks for 30 minutes, cooled in water at room temperature for 2 minutes, and centrifuged for 5 minutes at 10,000 \times g. One hundred μ L of the supernatant aliquot was transferred into another plastic centrifuge tube, and 20 μ L of 40 % trichloroacetic acid was added. The mixture was heated at 85°C in heating blocks for 15 minutes, centrifuged for 5 minutes at 10,000 \times g at room temperature. 200 μ L of 1 mM ferrozine solution (1 mM ferrozine in 1.05 M sodium acetate, pH 4.8) was added to 20 μ L of the supernatant aliquot. The absorbance was measured at 562 nm using spectrophotometer (Beckman DU40 spectrophotometer (Beckman Coulter, ON, Canada)).

6.4 Statistical analyses

Descriptive statistics were performed for the variables. Results were expressed as mean \pm standard deviation (SD), and analyzed using parametric test (one-way ANOVA and post hoc test Student-Newman-Keuls). A $p < 0.05$ was considered statistically significant. The correlation between the variables was evaluated using Pearson's correlation coefficient.

6.5 Results

6.5.1 Aluminum and iron concentration in serum

After two weeks of PN administration, the elevation in serum Al in the regular PN group was statistically significant ($p < 0.05$) on day 14 compared to the low aluminum PN group (Table 6.1). For serum iron concentration, the mean iron content in the regular PN group was higher than in the low aluminum PN group on days 7 and 14, but only on day 14 was it significant ($p < 0.05$) (Table 6.2). In the regular PN group, the increase in serum Al was significantly correlated ($r = 0.731$, $p = < 0.001$) with serum iron (Figure 6.1).

Table 6.1. The concentration of serum aluminum in piglets during the study.

Group	Aluminum (µg/L)			
		Day 0	Day 7	Day 14
Low Aluminum PN	mean±SD	320±261	504±484	218±185 ^a
	n	6 ¹	7 ²	7 ²
Regular PN	mean±SD	181±119	925±1631	884±630 ^b
	n	7 ³	7 ³	7 ³

Data was expressed as mean ±SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($p<0.05$). Means with different Superscripts (a, b) were significantly different ($p<0.05$) when compared to each.

1. Low aluminum PN group had two samples lost (piglet # 10, 28) for day 0.
2. Sample with insufficient serum (piglet # 28, day 7 and piglet # 25, day 14)
3. Regular PN group had three samples lost (piglet # 15) for day 0,7,14.

Table 6.2. The concentration of serum iron in piglets during the study.

Group		Iron (µg/L)		
		Day 0	Day 7	Day 14
Low Aluminum PN		2348±1072	4701±3025	6014±2318 ^a
	n	6 ¹	7 ²	7 ²
Regular PN		2542±1111	6332±3285	9290±3583 ^b
	n	7 ³	7 ³	7 ³

Data was expressed as mean ±SD. One-way ANOVA and Post Hoc test (Student-Newman-Keuls) was used to compare means at significance level ($p<0.05$). Means with different superscripts (a, b) were significantly different ($p<0.05$) when compared to each.

1. Low aluminum PN group had had two samples missed at day 0 (piglet 10&28).
2. Sample with insufficient serum (piglet # 28, day 7 and piglet # 25, day 14)
3. Regular PN group had serum samples (piglet # 15) for day 0,7,14 lost.

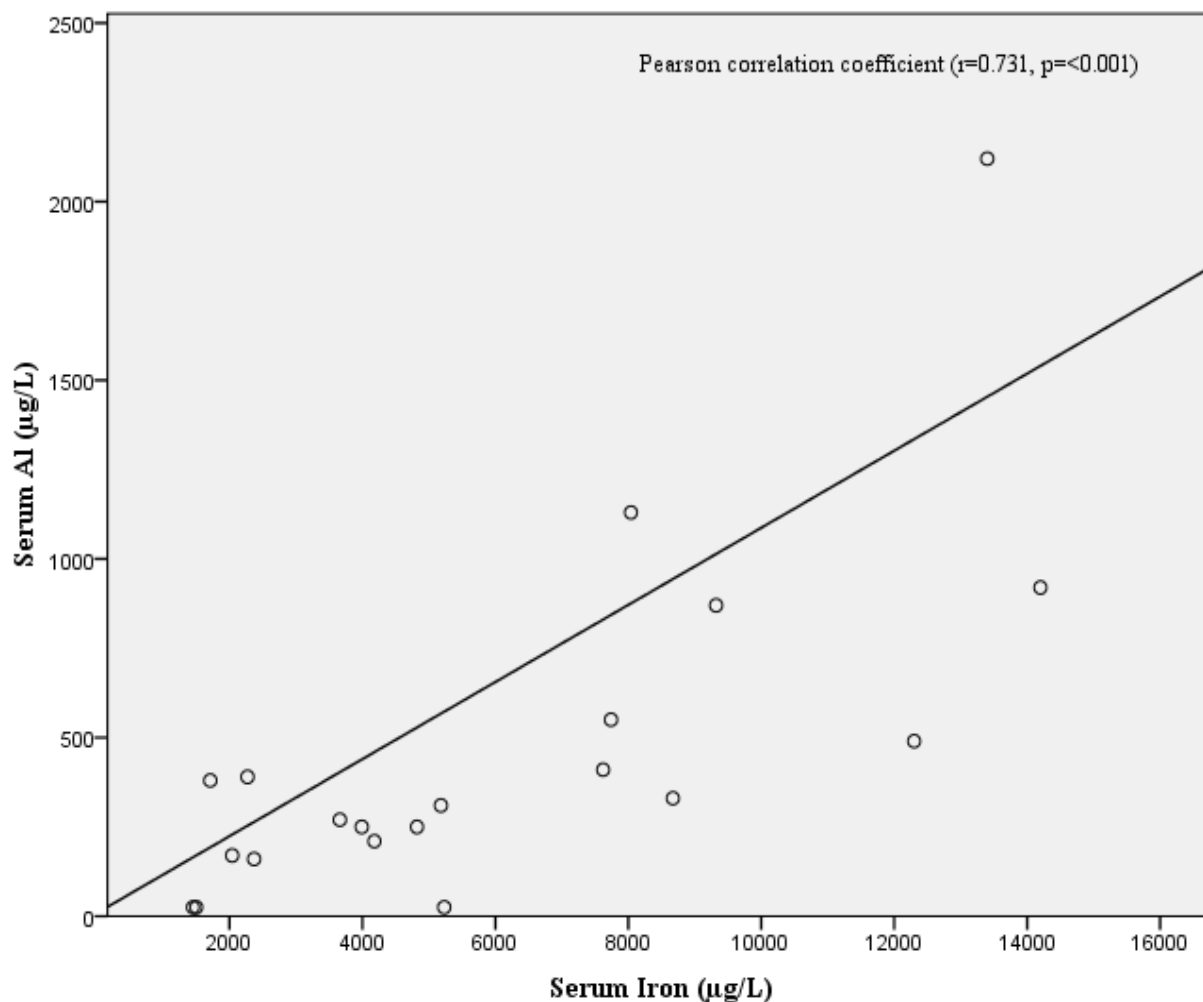


Figure 6.1. Correlation between serum aluminum and serum iron in the regular PN group.

6.5.2 Aluminum and iron concentration in liver

The mean hepatic aluminum content in the low aluminum PN group was significantly ($p<0.05$) lower compared to the regular PN group (Table 6.3). The mean hepatic iron content in the regular PN group was higher than the low aluminum PN but it wasn't significant (Table 6.3). The hepatic Al level was significantly correlated with non-heme iron and total hepatic iron (Table 6.4).

Table 6.3. Mean hepatic concentration of aluminum and iron in the piglets.

Group	Aluminum (µg/g)	Iron (µg/g)
Low Aluminum PN	0.30±0.20 ^a	237±78
n 6 ¹		7 ²
Regular PN	0.77±0.18 ^b	268±62
n 8		8

Values are expressed as means ±SD. Means with different superscripts (a, b) were significantly different (p<0.05) when compared to each other by one way-ANOVA/Student-Newman-Keuls test.

1. Low aluminum PN group had one sample missed (piglet # 6) and one sample insufficient for analysis (piglets # 22).
2. One liver sample (piglet # 6) missed.

Table 6.4 Correlation between hepatic aluminum and hepatic levels of TBARS, non-heme iron, bleomycin detectable iron, and total iron.

Parameter	Low aluminum PN		Regular PN	
	r	p-value	r	p-value
TBARS	0.440	0.229	0.440	0.162
Bleomycin Detectable iron	-0.449	0.448	0.387	0.195
Non- heme iron	0.294	0.315	0.825	0.011
Hepatic iron	0.349	0.249	0.809	0.008

P-value < 0.05 considered significant

r: Pearson correlation coefficient

6.5.3 Measurement of bleomycin detectable iron, non-heme iron and TBARS levels in liver tissues

Concentration of bleomycin detectable iron, an indicator of the free iron pool (Gutteridge et al., 1981), was measured in the piglet liver tissues of all the groups (Table 6.5). Bleomycin detectable iron levels were decreased non-significantly in the low aluminum PN group by 25% compared to regular PN group (Table 6.5).

Non-heme iron levels in the piglet liver tissues were measured in the low aluminum PN and regular PN (Table 6.5). The levels of non-heme iron in the liver of low aluminum PN group were non-significantly decreased by 18% compared to regular PN group, ($p = 0.355$) (Table 6.5). Non-heme iron was significantly correlated with hepatic Al concentration in the regular PN group ($r = 0.825$, $p = 0.011$) (Table 6.4).

The oxidative stress TBARS assay which is used to detect malondialdehyde (MDA) level in samples was used to assess piglet liver tissues in the two groups; the low aluminum PN and regular PN group (Table 6.5). The MDA level in the low aluminum PN group was reduced non-significantly by 54% compared to regular PN group ($p = 0.116$) (Table 6.5).

Table 6.5. TBARS, non-heme iron, and bleomycin detectable iron levels in piglet liver*.

Group	TBARS (MDA nmol/mg)	Non-heme iron ($\mu\text{g Fe/mg tissue}$)	Bleomycin detectable iron ($\mu\text{g Fe/mg tissue}$)
Low Aluminum PN (n=7) ¹	1.1 \pm 0.4	0.9 \pm 0.2	0.26 \pm 0.11
Regular PN (n=7) ²	2.4 \pm 2.0	1.1 \pm 0.2	0.35 \pm 0.05
p-value	0.116	0.355	0.105

Values are expressed as means \pm SD. Groups were compared to each other using one way-ANOVA/Student- Newman- Keuls test ($p < 0.05$).

1. Insufficient sample (piglet # 2).

2. Insufficient sample (piglet # 9).

*liver was harvested at the end of the study (day 14).

6.6 Discussion

The main objectives of this study were to evaluate the effect of reducing the aluminum contamination of parenteral nutrition on liver iron homeostasis and to determine if reducing the aluminum content of PN would decrease the free iron pool in the liver and reduce the signs of oxidative stress in liver tissues.

This study demonstrated that compared to regular PN, the administration of a low Al PN to newborn piglets for 14 days caused a significant decrease in Al levels in serum and liver tissues, but no significant reduction in hepatic free iron oxidative stress.

It is known that prolonged administration of PN to infants and children is associated with liver injury and hepatobiliary dysfunction, but still the exact mechanism that leads to liver injury is unknown. Several PN studies conducted in different models of animals reported that oxidative stress plays an important role in developing liver injury (Sokol et al., 1996; Sola et al., 2002; Weinberger et al., 2002; Cai et al., 2006). However, the investigators could not identify the source of oxidative stress in their models.

Al has evidently been established as a hepatotoxic metal. In our study, Al contamination in the regular PN solutions provide Al intakes in the range of 38 µg/kg/d which exceeds the Food and Drug Administration recommended exposure limit of 5 µg/kg/d. It is clear that Al contamination in the regular PN solutions led to significant accumulation of Al in the piglet's liver of regular PN group. A similar finding of hepatic Al accumulation has been observed in animals administered with PN (Klein, 1993; Demircan et al., 1998).

Al is a non redox-active metal with a high pro-oxidant activity that has been shown to facilitate lipid peroxidation in the presence of free iron (Oteiza et al., 1993; Verstraeten et al., 1995; Ohyashiki et al., 1996; Verstraeten et al., 1997). In vitro studies using liposomal and brain microsomal systems, Al ions were reported to have stimulating effect on lipid peroxidation

induced by iron ions (Quinlan et al., 1988; Verstraeten et al., 1994). Interestingly, Al ions were found to increase oxidation of low density lipoproteins (LDL) in the presence of Fe but not Cu (Kapiotis et al., 2005).

In our study, no significant difference was observed in the levels of hepatic free iron, non-heme iron, and TBARS between the two groups; however, these levels were non-significantly higher in the regular PN group compared to low Al PN group. Various animal studies conducted on different animal models have shown that administration of PN resulted in significantly increased levels of lipid peroxidation products (a measure of oxidative stress) in the liver tissues (Sokol et al., 1996; Cai et al., 2006; Hong et al., 2007). The lack of statistical significance could be attributed to the small number of animals used in the current study, and with a larger sample size differences may be detected.

In summary, the present study indicates that the administration of lower aluminum content PN led to less aluminum loading in the serum and liver tissues. However, we did not find significant reduction in the levels of free iron, non-heme iron, and lipid peroxidation products in the low Al PN compared to the regular PN therapy.

7.0 ALUMINUM CONTENT IN TOTAL PARENTERAL NUTRITION CAUSES DOWNREGULATION OF KEY CANALICULAR TRANSPORTERS

7.1 Introduction

Parenteral nutrition-associated cholestasis (PNAC) predisposes patients to the development of irreversible liver injury (Teitelbaum, 1997; Beath et al., 1996; Moss and Amii, 1999). Cholestasis may either result from a functional defect in bile formation at the level of the hepatocyte or from impairment in bile secretion and flow at the bile duct level. Despite the recognition of illness for more than 30 years, the mechanism responsible for PNAC is unknown. In the past decade, research in the area has attributed the pathogenesis of some forms of cholestasis to mutations in members of ATP binding cassette (ABC) transporter family of proteins (Trauner et al., 1998; Balistreri et al., 2005) or to the inhibition of transporters involved in maintenance of bile flow. Other forms include drug-induced cholestasis caused by drug or its metabolites (Stieger et al., 2000; Funk et al., 2001; Fattinger et al., 2001; Bode et al., 2002). Hepatobiliary transport proteins are responsible for the transport of various components of bile into the hepatocytes and/or the bile canaliculi (Trauner et al., 1998). Exposure to cholestatic injury (e.g., drugs, hormones, pro-inflammatory cytokines, and biliary obstruction) results in reduced expression and function in hepatobiliary transport proteins. Changes in the expression of hepatobiliary transporters provide a crucial mechanism to regulate bile acid homeostasis and to prevent hepatic bile acid toxicity in hepatocytes during cholestasis. The basolateral transporters, sodium-taurocholate co-transporting polypeptide (NTCP) and organic anion-transporting polypeptide (OATP) are the major transporters for the uptake of bile acids and organic solutes from blood into the hepatocyte (Kullak- Ulick et al., 2000; Meier and Stieger, 2002). The canalicular transporters bile salt export pump (BSEP), multidrug resistance-

associated protein 2 (MRP2) and multidrug resistance protein (MDR3) are responsible for the excretion of bile salts, organic anions and phospholipids into bile (Trauner and Boyer, 2003). The nuclear receptors, farnesoid X receptor (FXR) and pregnane X receptor (PXR), also play an important role in the regulation of bile acid metabolism. High bile acid levels can activate FXR, which directly or indirectly induces Bsep expression (Ananthanarayanan et al., 2001; Plass et al., 2002). The canalicular transporters, BSEP and MRP2, (Trauner and Boyer, 2003) are responsible for elimination of bile acids and conjugated bilirubin and, hence, play an important role in the hepatic detoxification processes. In addition, these two transporters are involved simultaneously in hepatic excretion of diverse drugs and their metabolites into bile. Thus, functional disruption of BSEP and MRP2 may result in accumulation in the liver of materials that are toxic and cause liver injury.

Aluminum, a non-essential element, has been a known significant contaminant in the components of PN solutions (Stedman et al., 1985; De Vernejoul et al., 1985). In 2004, Gonzalez et al investigated the effect of aluminum exposure on biliary secretory function in a rat model and concluded that intraperitoneal administration of Al led to downregulation of Mrp2 and no specific mechanism through which Al exerts its deleterious effects was defined (Gonzalez et al., 2004). However, an involvement of Al-induced oxidative stress was suggested as a mechanism to cause liver injury (Gonzalez et al., 2007).

7.2 Hypothesis and Objective

We hypothesize that aluminum content in PN causes down-regulation of canalicular transport proteins, Bsep and Mrp2. The objective of this study was to investigate the effect of low aluminum and high aluminum PN (regular PN) on the mRNA expression of Bsep and Mrp2.

7.3 Material and Methods

Frozen liver tissues (flash frozen in liquid nitrogen and stored at -80°C) from Yucatan piglets were used in this study. The sixteen Yucatan piglets (3-4 days old) were obtained from a breeding herd facility at Memorial University of Newfoundland, St John's, NL. The piglets assigned randomly into; regular PN (8 piglets) and low Al PN group (8 piglets). The piglets from the regular PN group received PN formulation with aluminum contamination at 38 µg/kg/day, and piglets from the low Al PN groups received PN with reduced aluminum contamination to 6 µg/kg/day (section 5). Both groups were administered PN (either low Al or regular PN) for 14 days. At the end of the study, piglets were killed humanly, and samples of bile, blood, and liver were collected. Liver samples were snap frozen with liquid nitrogen and stored at -80°C. The liver tissues (n=11); 5 from regular PN and 6 from low Al PN were used for quantitative reverse transcription-polymerase chain reaction (QRT-PCR) analysis for the following apical (canalicular) transporters: Bsep, Mrp2. However, five liver specimens (3 from regular PN and 2 from low Al PN group) were not suitably preserved for QRT-PCR analysis. The Institutional Animal Care Committee at Memorial University of Newfoundland, and University of Saskatchewan's Animal Research Ethics Board approved all animal procedures and protocols used in this study.

7.3.1 mRNA expression levels of Bsep and Mrp2 in piglet's liver with regular PN and low aluminum PN solution.

7.3.1.1 Total mRNA Isolation and Quantitative RT-PCR Analysis.

Total mRNA was extracted from frozen piglet's liver tissues using RNeasy Mini Kits according to manufacturer instructions (Qiagen Inc., Mississauga, ON). The mRNA purity and quantity were determined spectrophotometrically by measurement at 260 nm and the

OD260/OD280 ratio, respectively, with a UV/VIS spectrophotometer (8453E, Agilent Technologies, Palo Alto, CA). Total RNA was stored at -80°C until analysis. Gene sequences for each transporter were obtained from the National Center for Biotechnology Information GeneBank (NCBI) and specific primers were designed using Primer3 software (Whitehead Institute for Medical Research) (Table 7.1). Quantitative RT-PCR (QRT-PCR) analysis was carried out using a QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 7300 Real-Time PCR system. The QRT-PCR protocol was carried out according to manufacturer's instructions. The protocol consisted of reverse transcription (1 cycle at 48°C, 30 minutes), PCR initial activation step (1 cycle at 95°C, 15 minutes), three-step thermal-cycling (50 cycles; denaturing at 94°C, 15 seconds, annealing at 60°C, 30 seconds, and primer extension at 60°C for 30 seconds), and a melt curve analysis from 65°C-95°C at 0.5°C/second.

7.3.1.2 Validation of the $2^{-\Delta\Delta C_T}$ Method.

QRT-PCR assays were initially optimized to give a PCR efficiency between 1.9-2.1 (as determined by a 4-point standard curving using serial dilutions of control RNA with a slope range of -2.9 to -3.5) and a single melt-peak corresponding to the appropriate PCR product as verified by 2% agarose gel electrophoresis. The reactions were further optimized for usage of the $2^{-\Delta\Delta C_T}$ method using β -actin as an internal standard. The amplification efficiency of each target and β -actin was determined by constructing a standard curve from C_T and RNA concentration. The target genes and β -actin were then amplified using same diluted samples. The ΔC_T were calculated (i.e. the difference between the target gene C_T and β -actin C_T). The slope from log RNA concentration versus ΔC_T was close to zero (<0.1). Only primers giving PCR amplification close to 100% and the relative efficiencies between the target and β -actin that were approximately equal were used in our experiment. Fold differences in mRNA expression

between control and treated samples were then calculated.

Table 7.1. Primer sequence for RT-PCR of Bsep, β -actin and Mrp2.

Gene	Accession number	Primers	
		Forward	Reverse
Bsep (pig)	SSU20587	tcaccagcactttctcc	tgaccttggtccagttcg
Mrp2 (pig)	NM000392	ctgcaactgggttggtcc	catgatgggtgtgcagtcg
β -actin (pig)	DQ845171	gtggcaccacatgtacc	cgtcgtactcctgcttgc

Bsep: bile salt export pump; Mrp2: multidrug resistance-associated protein 2; β -actin: beta-actin.

7.4 Results

7.4.1 Evaluation of the expression of Mrp2 and Bsep in piglet liver.

The effect of regular PN and low aluminum PN solutions on canalicular transport proteins, Mrp2 and Bsep, were tested using QRT-PCR. In the regular PN group, the Mrp2 mRNA expression levels were significantly ($p < 0.05$) reduced compared to low aluminum PN group (Figure 7.1). Expression levels of Bsep mRNA could not be analyzed, as the Bsep primer used could not be validated. This could be attributed to using SYBR® Green dye assay (Applied Biosystems, Foster City, CA). The disadvantage of this dye is its lack of specificity as it detects all amplified double-stranded DNA sequence, including non-specific reaction products (Hardikat et al., 2014). Using a probe such as TaqMan assays (a fluorophore-tagged probe-based chemistry) which detects only specific amplification products, may help to overcome this problem and produce reliable results. Future work includes designing and validating another Bsep primer.

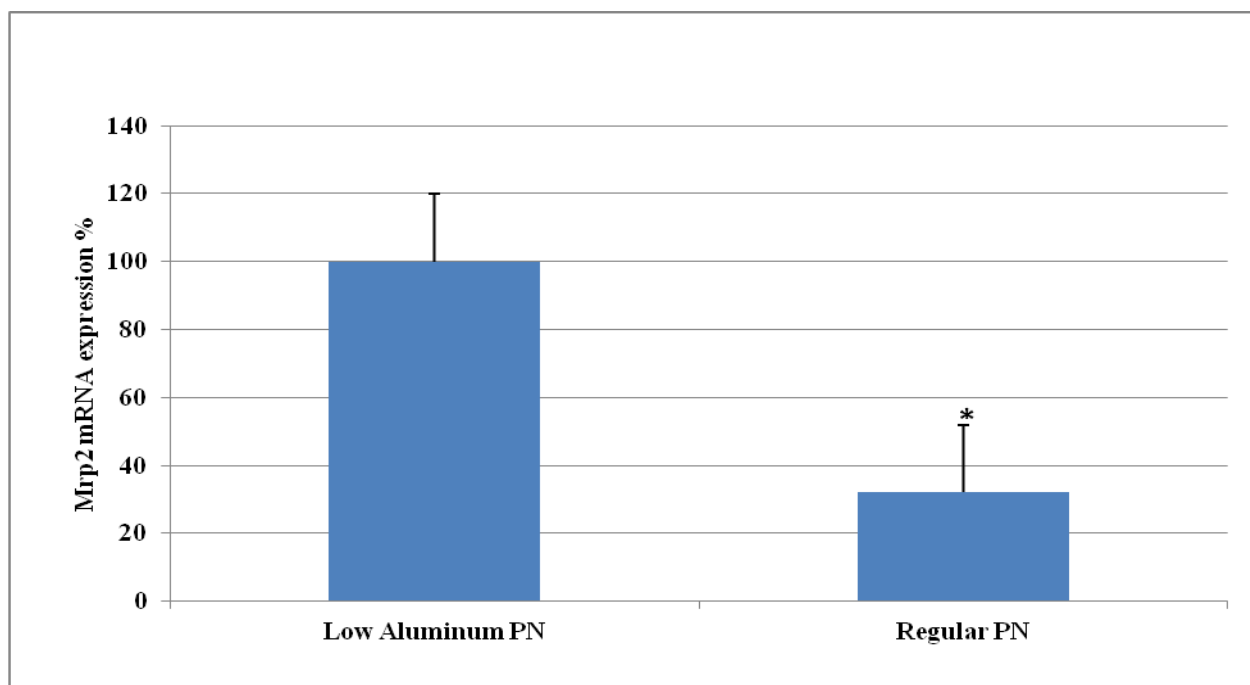


Figure 7.1. Expression levels of Mrp2 mRNA in piglet liver of both Low Aluminum and Regular PN group measured using QRT-PCR.

Regular PN group (n=5) received continuous PN infusion with aluminium contamination at 38 $\mu\text{g/kg/day}$ for 14 days. The low aluminum PN group (n=6) received continuous PN infusion with aluminum contamination at 6 $\mu\text{g/kg/day}$. * indicates that using independent t test, decrease in Mrp2 expression of regular PN group is significantly ($p < 0.05$) different compared to low aluminum PN group.

7.5 Discussion

In the present study, the effect of low and high Al content PN on the canalicular transporter Mrp2 was investigated by measuring mRNA expression levels in the liver of the Yucatan neonatal piglets after 14 days of PN administration.

In our previous work, we showed that administration of regular PN to newborn piglets for 14 days resulted in high hepatic and serum aluminum levels. The elevation was associated with liver injury evidenced by the loss of canalicular microvilli. These results supported our main hypothesis of the study that Al content could play an important role in causing liver damage, and encouraged us to proceed in our work to study molecular changes, specifically the bile canalicular transport system, and how it is affected by the administration of regular PN as compared with the low Al PN.

The overall results of the this experiment showed that regular PN infusion for 14 days caused downregulation of the Mrp2, an efflux transporter, in the liver of the neonatal piglets. In the literature, few studies investigated bile canalicular transporters in PN animal models. There are conflicting results about the mRNA expression levels of canalicular transporters in TPN administered animals. For example, a study on adult mice (10-12 weeks) administered with TPN for 7 days showed a decline in the mRNA expression of *mdr2*, but no significant changes in *mrp2* and *bsep* (Tazuke and Teitelbaum, 2009). In another study, three weeks old rats received TPN for 4 days had significant declines in the mRNA expression levels of Mrp2. The etiology behind the changes observed in this study was unknown (Nishimura et al., 2005); however, in a study for the effect of Al loading on canalicular transporters reported that Al could alter the bile canalicular transporter proteins and cause downregulation of Mrp2 (Gonzalez et al., 2004; Gonzalez et al., 2009). However, the authors suggested that Al exerts its toxic effects through oxidative stress.

In our study, although the TBARS levels, a marker of oxidative stress, were high in liver tissues of the regular PN group compared to low AI group, these levels did not reach statistical significance. Therefore, we cannot claim that the downregulation of Mrp2 seen in our study was associated with oxidative stress.

The underlying mechanisms by which AI contamination in regular PN resulted in the downregulation of Mrp2 are far from being understood and cannot be addressed by our results. Nonetheless, our experiment indicates that AI content in the regular PN might be involved in the downregulation of Mrp2.

Generally, in injured livers, the expression levels of basolateral transporters (uptake transporters) are reduced, and the levels of canalicular transporters (efflux transporters) are increased as a compensatory mechanism to maintain the bile flow and protect the liver from further injury (Trauner and Boyer, 2003; Slitt et al., 2007). In our study, it is possible that the reduced levels of Mrp2 with increased total bile acids reflecting the development of cholestasis in the neonatal piglets (Nishimura et al., 2005).

In conclusion, our results show that regular PN administration to newborn piglets for 14 days caused decreased mRNA expression of the bile canalicular transporter Mrp2. It is likely that AI content in PN plays a major role in the downregulation of Mrp2, however, in this study, using our results we could not determine the exact mechanisms that led to the downregulation of Mrp2. More research is needed to explore the underlying mechanisms that cause downregulation of bile canalicular transporter proteins during administration of parenteral nutrition in neonates.

8.0 General conclusion and future studies

The overall aim of this dissertation was to investigate the role of aluminum as a toxic component of PN and as a risk factor in developing parenteral nutrition associated liver injury. The study objectives were carried out using a variety of biochemical, histological, and biological imaging techniques to test our hypotheses.

Using a pig model, we have demonstrated that parenteral aluminum administration results in significant deposition of aluminum in the liver. This resulted in hepatocyte injury as demonstrated by significant increases in serum total bile acid levels. Interestingly, this was not reflected in a significant increase in serum direct bilirubin which is the biochemical marker used in clinical practice to indicate cholestasis. It may be that the duration of aluminum exposure was not sufficient to cause an increase in bilirubin. This may also be a reflection of the experimental model. We used newborn pigs born at full term gestation. We know that premature infants are at a much greater risk for parenteral nutrition associated cholestasis. Furthermore, aluminum is primarily excreted by the kidneys and the findings of our study may be confounded by relatively mature renal function compared to that seen in premature infants.

A troubling issue in this study was the finding of serum aluminum levels on day 0 that were markedly different between the study and control groups. We have not found a satisfactory answer for this difference. This may be a reflection of specimen contamination. Aluminum is ubiquitous in nature and there is always the potential of contamination during the animal care as well as the handling and processing of specimens. The study subject numbers were very small so outliers may confound the results due to statistical errors.

It is particularly significant that we discovered parenteral aluminum caused marked damage to the bile canaliculi microvilli. This is the anatomical location of the bile acid

transport proteins, Mrp2 and Bsep, that are critical to the normal bile physiology. This canalicular microvilli damage seen with aluminum administration mirrors that which has been seen in association with PNAC (Shu et al., 1991; Moran et al., 2005; Cai et al., 2006), and this finding helps to support our hypothesis that aluminum contaminating PN is a contributing factor in PNAC. The pathophysiology of how aluminum causes the canalicular microvilli damage is unclear. It is also unclear if the rise in serum bile acids is a reflection of the microvilli damage or if other mechanism might be at play. It is possible that the intracellular accumulation of bile acids causes a downregulation in the expression of those genes responsible for the production of these bile acid transport proteins. We recognize that the dose of aluminum used for these studies was far in excess of that seen in PN therapy however we wanted to first demonstrate the toxic effects of aluminum and in our later work we would evaluate aluminum in doses that reflected that seen in clinical practice.

In trying to understand the pathophysiology of the hepatic toxicity of aluminum, we were interested in oxidative stress. Aluminum competes with iron for binding to transferrin and we were interested if the aluminum was negatively effecting iron homeostasis leading to the production of toxic free radicals. Contrary to our expectations, the significant elevation in Al levels in the serum and liver tissues of piglets of all study groups did not cause a significant increase in the levels of bleomycin detectable iron and oxidative stress as determined by measuring the lipid peroxidation by-product, TBARS in the liver tissues. There was an increasing trend in the hepatic levels of free iron and TBARS and this may reflect the disruptive effect of Al loading on tissue iron homeostasis. At this time, we can only speculate that Al disrupts iron homeostasis and plays a role in causing oxidative stress through its disrupting effect on iron homeostasis or its pro-oxidant effect resulting in increased tissue free iron thus

stimulating lipid peroxidation leading to liver injury. Our results don't support this however, it is possible that a larger sample size may help to conclude whether Al loading disrupts iron homeostasis in the liver tissues or not.

Looking at the issue of the aluminum induced canalicular microvilli damage seen in our earlier work we then approached the question; if we reduce the amount of aluminum in PN would it result in less microvilli damage? To study this we chose to use a newborn Yucatan miniature piglet model. These animals grow at a slower rate than the previous study pigs and more closely mimic the growth rate of a human infant. We administered PN to one group with the aluminum contamination level typical of that seen in human infant PN therapy. This was compared to a group receiving PN with a much lower aluminum contamination level. After 14 days the serum and hepatic aluminum levels were much lower in the low Al exposure group. This correlated with a significant reduction in the degree of microvilli damage. This was encouraging as it seemed to support our hypothesis. These results were not reflected in the serum total bile acid levels. We expected to see a higher serum bile acid level in the serum of piglets with greater aluminum exposure. While there may be a trend in this regard the differences were not significant. Our subject numbers may have been too low to demonstrate a real difference or it may be that a longer duration of aluminum exposure is required to show a significant result. Our study was only 2 weeks duration and clinically significant liver injury may take longer to manifest. It may be that the canalicular microvilli damage is the earliest evidence of the hepatotoxic effects of aluminum.

We found there was less serum and hepatic accumulation of Al in the low aluminum group compared to the regular PN group. As we expected, the reduction in the serum and hepatic Al content was associated with lower serum total bile acids in the low aluminum PN

group compared to regular PN group. These findings support our hypothesis that reducing aluminum content in the PN solutions reduces liver injury in the piglets administered with PN. Also, it indicates that Al might, in part, be responsible for the liver injury seen in piglets infused with PN. Moreover, this study's results showed that reducing Al content in PN solutions has led to significant reduction of Al accumulation in the serum and liver of newborn piglets. This reduction was associated with less morphological changes and damage in the bile canaliculi microvilli.

It has been demonstrated in other types of cholestasis that there is a reduction in the hepatic expression of mRNA for the bile transport proteins Mrp2. We were interested in seeing if aluminum had a deleterious effect on the expression of the bile acid transport proteins Mrp2 and Bsep. Due to technical challenges we were not able to obtain results for Bsep. We did find that the higher aluminum exposure in PN resulted in a greater reduction in the expression of Mrp2. This may help to explain at least one mechanism by which aluminum may contribute to PNAC. Unfortunately, our study did not demonstrate a corresponding rise in serum bile acid levels. Again, this may be a reflection of small subject numbers or it may be too short a study period to demonstrate a rise in bile acids. The decrease in Mrp2 expression may be an early sign of the hepatotoxic effect of aluminum.

8.1 Future study

This work has discovered that aluminum in PN significantly accumulates in the liver and this is associated with both canaliculi microvilli damage and a reduction in the expression of the bile acid transport protein Mrp2. From these findings there are opportunities to expand these investigations into the potential role of aluminum as a contributing factor in PNAC. Additional work to look at the potential for aluminum to lead to cholestasis through oxidative stress is

worthwhile. Employing a greater sample size or using a different research model may help to reveal a true role for oxidative stress in the pathophysiology of PNAC and a potential for antioxidant therapy in the prevention and treatment of this disease.

While we found that aluminum caused damage to the canalicular microvilli how this occurs and the mechanisms involved deserves further study. Some types of cholestatic disease have demonstrated that bile acid transport proteins are displaced from the microvilli apex surface to a more sub-apical location. Radixin is the dominant ezrin-radixin-moesin protein in bile canalicular membrane, and a cross-linker between actin filaments that forms the cores of canalicular membranes and plasma membrane proteins, and known as an important component in the formation of canalicular microvilli of hepatocytes (Amieva et al., 1994; Kondo et al., 1997). A study on mice lacking radixin demonstrated that the mice develop conjugated hyperbilirubinemia associated with loss of microvilli and Mrp2 from the canalicular membrane, indicating that radixin is required for the secretion of conjugated bilirubin through the efflux transporter Mrp2 (Kikuchi et al., 2002). A future study investigating the effect of Al in PN on radixin may elucidate the mechanisms behind the loss of canalicular microvilli and downregulation of Mrp2 in the piglet's liver.

The effect of aluminum on the downregulation of the bile acid transport proteins is open for more investigation. We could look at what levels this is occurring such as mRNA level, initiation of transcription, and promoter activity. This will help to unravel the molecular mechanisms involved not only in the hepatotoxicity of aluminum but also of other factors in PNAC .

Outside of the role of aluminum the effect of different fat emulsions specifically Omega-3 based versus Omega-6 based is the current area of most interest in understanding PNAC. The

anti-inflammatory nature of Omega-3 fatty acids has been shown to reduce the cholestasis of PN therapy. Our work with imaging of the canalicular microvilli may prove very useful in evaluating the effects of these different fat emulsions in relationship to PNAC. Likewise the study of the molecular expression of bile acid transport proteins with the different fat emulsions may help to further understand PNAC including how to prevent and treat it.

9.0 References

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10.0 Appendices

Appendix A

Table 10.1 Oral nutrient requirements for piglets (NRC, 1998).

Nutrient	Requirement*
Metabolizable energy, kcal	820
Amino acids, g	65.0
Essential amino acids, g	24
Dextrose, g	ND
Sodium, mmol	27.4
Potassium, mmol	19.2
Calcium, mmol	56.1
Magnesium, mmol	4.1
Chloride, mmol	17.7
Phosphate, mmol	56.5
Zinc, μ mol	382
Manganese, μ mol	18.2
Copper, μ mol	23.6
Chromium, μ mol	ND
Selenium, μ mol	1.0
Iodine, μ mol	0.3
Iron, mg	25.0
Cobalamin, μ g	5.0
Biotin, μ g	20.0
Retinol, μ g	189
Ergocalciferol, μ g	1.4
α -Tocopherol, mg	2.7
Phylloquinone, μ g	130
Ascorbic acid, mg	ND
Thiamin, μ g	380
Riboflavin, μ g	1000
Pyridoxine, μ g	500
Pantothenate, mg	3.0
Folate, μ g	80
Niacinamide, mg	5.0
Lipid g	32

* based on piglets weighing 3 to 5 kg